OXIDATIVE STRESS AND SEED SURVIVAL

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"A single exposure to this element causes lifelong addiction, withdrawal for more than a few minutes can be fatal, there is no cure, and the prognosis is invariably death. The element is called oxygen."

John W. Campbell Jr.

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Abstract

Free radical and aldehydic breakdown product content were determined, by EPR and UV / visible spectroscopy, primarily in intermediate (desiccation tolerant) seeds of *Carica papaya* L. (Papaya) and recalcitrant (desiccation intolerant) seeds of *Aesculus hippocastanum* L. (Horse chestnut), but also in other species covering a range of desiccation tolerances, with a view to determining the role of oxidative stress as a diagnostic marker for desiccation tolerance.

Axes of non-senescent highly viable recalcitrant seeds of horse chestnut were metabolically active, contained products of lipid peroxidation, displayed low levels of enzymatic protection against activated oxygen and peroxides, and a two-peak free radical EPR signal. During fully hydrated storage at 16 °C for up to 18 months, seeds exhibited, sequentially, an increase in germination rate, a transient increase in intensities of both the low field and high field EPR peaks, a significant increase in membrane leakage and decrease in seed viability, germination rate, and SOD and peroxidase activities. Drying 'unstored' seeds below and embryonic axis moisture content of 40 to 50 % initiated viability loss. At < 25 % moisture content all axes were inviable and displayed a 2- to 4-fold increase in solute leakage, lipid peroxidation products and the low field EPR signal. Seed desiccation sensitivity increased with hydrated storage. The accumulation of lipid peroxidation products and free radicals on drying generally occurred to a greater extent, or at a higher moisture content, than observed with unstored seeds. The results indicate a mediating role for oxidative stress in recalcitrant seed viability loss which is differentially expressed during hydrated, 'natural' ageing and desiccation. Similar trends were seen in other recalcitrant species with the increase in lipid peroxidation products occurring around the point of viability loss. However the study of a more orthodox species (papaya) revealed no such trends.

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ABA	Absiscic Acid
ANOVA	Analysis of Variance
atm	One atmosphere of pressure
BSA	Bovine Serum Albumin
BHT	Diboron Tetrahydroxide
°C	Degree Celcius
d	Days
DMSO	Dimethylsulphoxide
DTPA	Diethylenetriaminepentaacetic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DNA	Deoxyribonucleic acid
DSC	Differential Scanning Calorimetry
d. wt.	Dry weight
EDTA	Ethylene diaminetetra acetic acid
EPR	Electron Paramagnetic Resonance
eRH	Equilibrium Relative Humidity
ESR	Electron Spin Resonance
f. wt.	Fresh weight
G	Gravitational force
GC	Gas Chromatography
GC - MS	Gas Chromatography - Mass Spectroscopy
GSH	Glutathione
GSSG	Reduced Glutathione
HF	High Field
4-HNE	4 - Hydroxy-2(E)-nonenal
HPLC	High Power Liquid Chromatography
$J\ g^{-1}$	Joules per gram
K	Kelvin

kHz	kiloherz
µl	microlitre
LEA proteins	Late Embryogenesis Abundant proteins
LF	Low Field
LPO	Lipid Peroxides
M	Molar
MCDP	10-N-Methylcarbamoyl-3,7-dimethylamino-10H-phenothiazine
MDA	Malonaldehyde
ml	Millilitre
mM	Millimolar
mm	Millimetre
MPa	Mega Pascals
$m\ s^{-1}$	Metre per second
mT	Millitestla
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised)
NAPH	Nicotinamide adenine dinucleotide phosphate (reduced)
nm	nanometres
nmol/ml	nanomoles per millilitre
NMR	Nuclear Magnetic Resonance
OD	Optical Density
psi	Pounds per square inch
R1	Reagent 1
rpm	Revolutions per minute
RSH	Mercaptans (e.g. Glutathione)
S	Standard
s.d.	Standard Deviation
SDS	Sodium Dodecylsulphate
SOD	Superoxide Dismutase
TBA	Thiobarbituric Acid

TBARS	Thiobarbituric Reactive Substances
TCA	Trichloroacetic Acid
TLC	Thin Layer Chromatography
TZ	tetrazolium salt
UV	Ultra Violet
μW	Micro Watt
$W\ m^{-2}\ s^{-1}$	Watts per metre squared per second
w / v	Weight / Volume
v / v	Volume / Volume

For Laura,

and to the memory of the late

John W. Winskill

CHAPTER 1

General Introduction

Chapter 1: General introduction

Outlay of Chapter

Chapter 1: General Introduction

1.1 Seed banks: their use in conserving biodiversity

Seed banking is a relatively simple method of conserving plant genetic resources on an *ex situ* basis. Seeds are arguably the most convenient, practical and economical part of the plant to store. Many are relatively small, and are by their very nature adapted for storage and dispersal. They provide the most natural vehicle for the collection, transport and storage of genetic information, often avoiding the technical difficulties and expense inherent in other methods. Moreover, a large amount of genetic variation can be stored in a relatively small space. Seed banks exist to conserve and provide material for exchange, to anyone who can make efficient use of it. This in turn it is hoped may facilitate the re-introduction of species that become threatened in the future (Prendergast *et al.*, 1992), thus supporting *in situ* conservation techniques (restoration ecology / land reclamation).

Current recommendations for seed storage are between 3 - 7 % moisture content (species dependent) at -18 °C or less in air tight containers (FAO / IPGRI, 1994). Under such conditions it is predicted that seeds may survive for many decades. The accessions are periodically checked for viability, and regeneration is initiated when the germination percentage falls below a critical value which is species dependent.

1.1.1 Fundamental aspects of seed storage

Studying seed viability and / or its maintenance during storage has relevance to a number of areas. Although it is not normal agricultural practice to store seeds for long periods of time before use, viability loss during storage is a significant problem in regions of the world with climates which hasten seed deterioration (Minor and Paschal, 1982); this is also applicable to situations in which the seed is exported from its country of origin (Hampton and Bell, 1989); and where the market for a seed crop exhibits a large degree of variability (Mosjidis, 1991). In addition, seed storage has become an increasingly important area of germplasm conservation, where the genetic material of both wild and cultivated plant species are stored as seeds within seed banks (Chin, 1994). Hence, the problems associated with seed viability upon storage have a dramatic impact upon species conservation.

The study of longevity of seeds under differing storage environments is not a new subject and has long fascinated man, as reviews of early literature by Bewley and Black (1982) and Priestly (1986) show. Major reviews by Ewart (1972) and Harrington (1972) have attempted to classify species in respect to differences in absolute longevity recorded in the literature, but such systems have now been shown to be flawed, as seed longevity is influenced by environment during both production and storage (Roberts, 1972, Bewely and Black, 1982 and Priestly, 1986).

1.1.1.1 *Classification of seeds based upon their storability characteristics*

Roberts (1973) classified seeds according to their storage behaviour. Desiccation tolerant seeds are termed 'orthodox', and their longevity is seen to increase if dried

to low moisture contents (approximately -350 Mpa; Roberts and Ellis, 1989) and by decreasing temperature and humidity (Roberts, 1973 and Roberts and Ellis, 1989). This storage behaviour can be quantified using a species specific seed viability equation (Ellis *et al.*, 1989), allowing longevity predictions to be made over a range of moisture contents and temperatures (Roberts and Ellis, 1989 and Dickie *et al.*, 1990).

The viability equation,

$$v = K_i - \frac{p}{10}\left(K_E - C_W \log m - C_H t - C_Q t^2\right)$$

predicts probit percentage viability, v after p days at a storage temperature of t °C and a moisture content, m (on a f. wt. basis). K_E, C_W, C_H and C_Q are constants (Ellis, 1988). K_i is also a constant, but not in the same sense that the others are, K_i is seedlot dependent, whereas the others are generally thought to be species specific. K_E and C_W define the way in which longevity alters with moisture content, C_H and C_Q define the temperature dependency of the response.

In addition to these responses, it is now clear that, providing they remain dormant, orthodox seeds are also capable of surviving for long periods at ambient temperatures when fully imbibed either continually or intermittently (Roberts and Ellis, 1989).

However, whilst 'orthodox' seeds form an effective means of *ex situ* conservation for many species, these storage conditions are not suitable for all species, simply because they do not produce seed which can survive the drying process. Several exciting techniques are being developed for the storage of desiccation intolerant seed, such as cryo-preservation (see Pritchard *in* Day and McLellan, 1995), but these have still to be widely accepted as a economical, safe, and viable alternative to long-term seed germplasm storage.

Seeds that do not survive desiccation, are termed 'recalcitrant' (King and Roberts, 1979 and Chin, 1988), and are intolerant of drying below a comparatively high moisture content (Roberts, 1973). This is species dependant, but falls within a range of 12 - 31 % (Roberts *et al.*, 1984, Chin, 1988 and Roberts and Ellis, 1989). Moreover, even at higher moisture contents (close to fully imbibed) they tend to exhibit comparatively short longevity (Roberts *et al.*, 1984), due to germination and / or fungal attack (Chin, 1988). There is also evidence that recalcitrant seeds suffer from chilling damage below c. 15 °C (King and Roberts, 1979a, 1979b, 1979c and Chin, 1988). Taxa with recalcitrant seeds often include tropical species such as *Theobroma cocoa*, some of the Araucariaceae spp. and the Dipterocarpaceae spp. (Tompsett and Kemp, 1996), which thrive in humid conditions. However, recalcitrant seeds are not always tropical and can include temperate species found commonly in the U.K. e.g. *Aesculus hippocastanum* L., *Quercus robur* L., and *Quercus rubra* L.

Robert's terminology has now been widely adopted and work on recalcitrant seeds by Probert and Longley, (1989), Roberts and Ellis, (1989), Dickie *et al.*, (1991) and Pritchard, (1991) has identified a range of seed water potentials to which recalcitrant seeds can be dried (-1.5 to -5.0 MPa, species dependent). Alternatives to the terms orthodox and recalcitrance have been suggested, for example 'poikilohydric' and 'homoiohydric' (Berjak *et al.*, 1990). However, in the latter case there is no evidence that the seeds are actually capable of withstanding / avoiding desiccation by maintaining their hydration status, which the term 'homoiohydric' infers. It has also been suggested that recalcitrant seeds could be further categorised into three groups - *minimally*, *moderately* and *highly* recalcitrant (Farrant *et al.*, 1988).

In addition to orthodox and recalcitrant, a third category of seed storage behaviour has been put forward (Ellis *et al.*, 1990, 1991). In these 'intermediate' seeds longevity is increased by a certain amount of desiccation, but too much results in reduced viability and longevity at low (0 to -20 °C) temperatures (Ellis *et al.*, 1990, 1991a, 1991b and Ellis *et al.*, 1991). Examples of intermediate seeds are *Coffea arabica* L. (which can withstand desiccation to c. 10 % moisture content, but further desiccation to c. 6 % is immediately damaging; Ellis *et al.*, 1991), *Carica papaya* L. (showed desiccation tolerance to 9.8 % moisture content, and reduced longevity at 0 °C; Teng and Hor, 1976) and oilpalm (no sub-zero tolerance, but viability can be maintained at 10 - 12 % moisture content at 15 °C; Ellis *et al.*, 1991). Rehydrated orthodox seeds can exhibit storage behaviour similar to that of intermediate seeds, but it is distinguished in its behaviour as it is only expressed once germination has started.

1.1.1.2 *Differences and similarities between orthodox and recalcitrant seeds*

Seeds display what has been termed 'adaptive survival', and seed technologists and banking staff can exploit the natural ability of seeds to survive stresses (which are usually secondary induced). Orthodox seeds, for instance, naturally desiccate and have become 'cold tolerant', recalcitrant tropical seeds on the other hand cannot tolerate low temperatures, and instead germinate immediately. If conditions are not adequate and orthodox seed dries they can enter a state of dormancy, a subject we will refer to in section 1.1.3 and Chapter 7. The major physiological difference between orthodox and recalcitrant seed development occurs during the acquisition of desiccation tolerance by orthodox species (Kermode, 1990). Orthodox seeds normally acquire tolerance to desiccation midway through development (Kermode, 1990), when levels of abscisic acid (ABA) are also quite high (Quatrano, 1986; Kermode, 1990 and Hetherington and Quatrano, 1991). Finch-Savage (1992) and Tompsett and Pritchard (1993) have also dealt with the development of seeds, and hypothesise that in contrast to orthodox seeds, recalcitrant seeds are generally shed prematurely (i.e. after shedding the germinability of seed lots can be improved by manipulating exogenous temperature and moisture conditions).

The regulatory processes governing the acquisition of desiccation tolerance by developing orthodox embryos are poorly understood, but could involve a combination of several factors; including the plant growth regulator ABA, carbohydrate accumulation, and the accumulation of specific classes of proteins which act as desiccation protectants in the cytosol (Kermode, 1990).

Orthodox seeds become desiccation intolerant once they have undergone germination processes which culminate with cell division and vacuolation (Bewley, 1979, Sargent *et al.*, 1981 and Perl, 1987). Desiccation damage can be characterised by increased cytoplasmic leakage of electrolytes upon rehydration, due to membrane damage (Chin *et al.*, 1981) and altered membrane permeability (McKersie and Stinson, 1980, McKersie and Tomes, 1980 and Senaratna and McKersie, 1983).

It appears that damage to membrane systems after drying accompanies viability loss in both recalcitrant seeds, and germinating orthodox seeds (which become desiccation intolerant).

However, it is not known why recalcitrant seeds are intolerant of desiccation. Berjak and co-workers have proposed that the desiccation intolerance exhibited by recalcitrant seeds is related to the initiation of germination (Berjak *et al.*, 1984, Farrant *et al.*, 1985, Farrant *et al.*, 1986, 1988 and Berjak *et al.*, 1990), and the further germination progresses, the more sensitive the seeds become to desiccation. This model assumes that there is no period of developmental quiescence (dormancy) in mature recalcitrant seeds, and germination commences when the seed is shed from the mother plant (Farrant *et al.*, 1986, 1988, 1989 and Berjak *et al.*, 1990).

1.1.2 Seed deterioration - loss of viability

Different species of seed take different periods of time to lose viability, but there are also variations in life span within species, and even within a 'seedlot' under the same moisture content and temperature, the term given to a nominally uniform population

of seeds (Roberts, 1972a). This variation can be seen when, for a population of seeds, percentage germination is plotted against storage time to produce a seed survival curve (Fig 1.1).

In the context of storage, individual seeds have a finite lifetime, and thus a point will be reached when the capacity to germinate is lost. However, many other symptoms of deterioration can be seen prior to the loss of germinability. These include decreased rate of germination, increased susceptibility to stresses, increased proportion of abnormal seedlings and increased leakage of electrolytes from imbibed seeds (Ellis and Roberts, 1981, Roberts and Ellis, 1982 and Roberts, 1986). It appears that seeds progress sequentially through these various stages of declining 'vigour' with, for example, the decrease in the rate of germination being one of the first measurable symptoms (Delouche and Baskin, 1973, Ellis and Roberts, 1981, Dell' Aquila, 1987 and Bradford *et al.*, 1993).

1.1.2.1 *Possible causes of seed deterioration*

A considerable amount of literature has described the complex changes which occur within seeds during deterioration. It is not the purpose of this section to describe them in detail, but the major causes of such events will be outlined. For more detailed descriptions of these events see, for example, Priestley (1986).

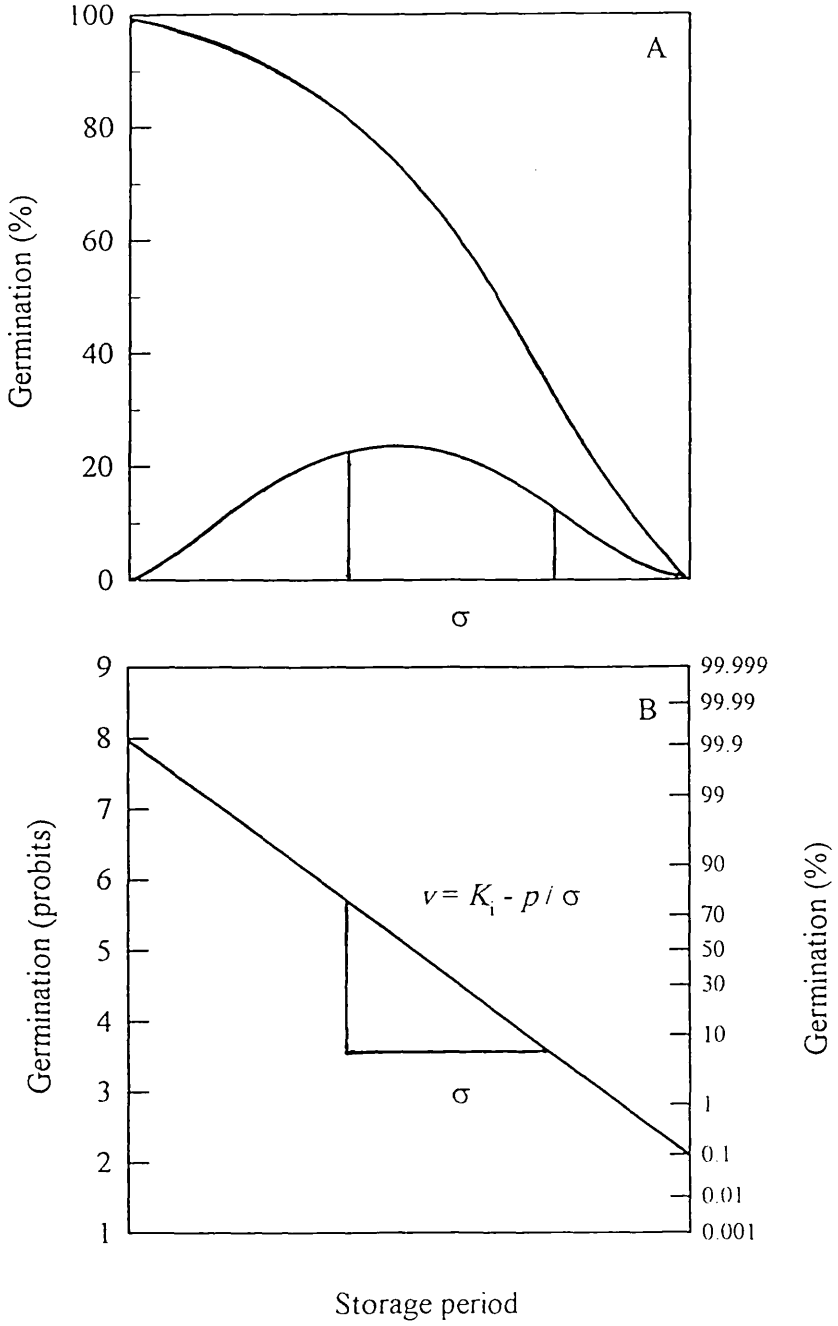


Figure 1.1 Seed survival curves: (A) the frequency distribution of seed deaths in time (lower curve), this gives rise to the negative cumulative normal distribution of seed deaths in time (upper curve), (B) the same curve plotted on a probability scale (right axis) or transformed to probits (left axis).

1.1.2.1.1 *Chromosomal aberrations*

Different types of treatment are commonly used to study ageing in seeds, artificial ageing is commonly used to accelerate the process and is achieved by exposing the seeds to combinations of high humidity and temperatures. It has been noted however, that when seeds undergo such high temperature treatments, damage can occur in the form of gross chromosomal aberration. In some cases point mutations also appear to increase in certain seeds upon ageing (Abdalla and Roberts, 1969 and Rao *et al.*, 1987), and this type of damage is not apparent in unaged seeds (reviewed by Barton, 1961, Roberts, 1972 and Roos, 1982 and 1984). This accumulation of chromosomal aberrations is related to the seeds storage conditions and points to a decline in the seeds viability (Roberts, 1967, Abdalla and Roberts, 1968, Murata *et al.*, 1981, Dourado and Roberts, 1984 and Rao *et al.*, 1987). However, chromosomal damage is not the ultimate cause of viability loss as quantitative differences between work, do exist (Rao *et al.*, 1988 and Lanteri and Belletti, 1990).

1.1.2.1.2 *Changes in enzyme activities*

It was once thought that loss of viability in seeds was due to a depletion of food reserves (Barton, 1961), however, this has long been discounted and it is now ascribed to the changes in enzyme activities. Free fatty acid production resulting from lipolytic activity, hydrolysis of phytin by phosphatases and proteolysis by proteases increase the acidity of the cell upon ageing (reviewed by Adbul-Baki and Anderson, 1972). Other hydrolytic enzymes have also been shown to increase upon ageing (Chastril, 1990 and Basavarajappa *et al.*, 1991). But it is usually, the activity of specific enzymes such as respiratory enzymes (Ching, 1973, Parrish and Leopold,

1978 and Dryer and Van der Venter, 1992), enzymes involved in the synthesis of polysaccharides (Anderson and Abdul-Baki, 1971), nucleic acids (Bray and Dasgupta, 1976 and Sen and Osbourne, 1977) and proteins (Hallam *et al.*, 1973 and Bray and Chow, 1976) that are seen to decrease upon seed deterioration. The decline in enzyme activity in certain cases may have occurred after cell death (MacLeod, 1952). In more recent studies Petruzelli and Taranto (1990) reported a decreased amylase activity in aged wheat, and Livesely and Bray (1991) have shown that the enzyme is synthesised in low levels by aleurone in aged seeds.

1.1.2.1.3 *Specific changes in lipid composition*

Seed ageing can be accompanied by an increased tendancy for the cells to leach solutes (Ching and Schoolcraft, 1968, Powell and Matthews, 1977, Stewart and Bewely, 1980, Ram and Wiesner, 1988 and Pandey, 1989). Seed viability is also seen to decline with the impaired function of membrane bound organelles, leading to a loss in membrane integrity (Parrish and Leopold, 1978, Powell and Harman, 1985 and Dawidowicz-Grezegorzewska and Podstolski, 1992). Studies have looked at the loss of membrane phospholipids during ageing (Pearce and Abdel Samad, 1980, Priestly *et al.*, 1980, Powell and Matthews, 1981, Halder *et al.*, 1983 and Fergerson *et al.*, 1990) and two mechanisms may account for the loss of lipids: (i) degradation by lipolytic enzymes (as discussed above); or (ii) lipid peroxidation (Priestly, 1986 and Wilson and McDonald, 1986) which is though to contribute to the ageing of animals (Finch and Hayflick, 1977). Lipid peroxidation is associated with many chemical intermediates, some of which (in particular free radicals) are important as they cause protein degradation, DNA damage and effect respiratory functions

(Wilson and McDonald, 1986). Free radicals and hydroxyalkenals, another product of lipid peroxidation, are mutagenic and are the likely causes of membrane damage and age related damage to genes (Reiss and Tappel, 1973). They also cause deficiencies in DNA repair mechanisms (Villiers and Edgcome, 1975 and Cheah and Osbourne, 1978) as DNA polymerase and DNA ligase activity (Vázquez-Ramos *et al.*, 1988), for example, is seen to decline as seeds age. Attempts to correlate viability loss with the preferential loss of unsaturated fatty acids, accumulation of free radicals and levels of free radical scavengers, have, thus far been inconclusive (reviewed by Bewley, 1986, Priestly, 1986 and Benson, 1990).

1.1.2.1.4 *Oxidative reactions*

Alterations associated with seed deterioration may be enyzmatically controlled. However, though there are many others that are clearly non-enzymatic such as the oxidative Amadori and Maillard reactions. In such reactions the aldehydic group of a reducing sugar reacts with the amino group of a protein, and they have been found to be related to the ageing of animals (Cerami *et al.*, 1987). Wettlaufer and Leopold, (1991) and Blackman and Leopold, (1993) have found an increase in the products of such reactions during the accelerated ageing of soybean seeds. Zhang *et al.*, (1995) and (1996) have proposed a similar modification of amino groups (and hence seed ageing), by volatile aldehydes evolved from the seeds themselves.

1.1.2.2 *So many possible mechanisms, which one to study ?*

It is clear that although many physiological and biochemical changes are known to be associated with seed deterioration, the primary cause of viability loss is still

unknown. At the start of the 90's emphasis was placed on the storage environment, particularly moisture content, during ageing as the key to understanding seed survival, based on the knowledge that water content affects the kinetics and nature of the reactions occurring in seeds (Leopold and Vertucci, 1989 and Vertucci, 1989).

However, it becomes apparent from the relative lack of literature that little effort (compared to other subject areas) had been put into determining the role of oxidative stress in seed survival, even though there is considerable evidence for an oxidative stress component to food quality deterioration (reviewed in St. Angelo, 1992), and the subject is now to be commonly found in medical papers, and the many subject specific journals. Hence, more recently this has been an area of more intense research.

We do not understand the effects of oxidative stress on the desiccation sensitivity mechanisms of seeds, or the differences (of the stress response) that may exist, if any, between desiccation tolerant and intolerant seeds. There is little information available as to the ability of seed cells to withstand the pernicious by-products formed during reactions involving activated oxygen species. It may also be possible that there are physiological and biochemical relationships progressing during oxidative stress, for instance, a link may exist between respiration rate and relative desiccation sensitivity. However, a majority of environmental stresses, natural and experimentally-imposed (i.e. heat shock, chilling, irradiation, desiccation and cryo-preservation) have been seen to induce oxidative stress in whole and / or plant part based studies. Thus, the focus of this investigation was to study the effects of

oxidative stress upon various aspects associated with seed storage. To serve as an introduction to this subject section 1.2 of this chapter will concentrate on the role of free radicals and lipid peroxidation in relation to seed quality, along with an introduction to, and the more general aspects of, oxidative stress and its measurement.

1.1.3 Seed Dormancy

In section 1.1.1.2 the term 'dormancy' was raised, which is in itself is an interesting aspect of seed biology, that has received considerable attention. Environmental conditions usually have to be very favourable for a seed to actually germinate. This can be related to temperature, light or gaseous concentrations. Seed usually remains viable (Bewley and Black, 1982 and Priestly, 1986) in a quiesent state, and germinates upon removal of the environmental constraint. In contrast to this, many seeds are still incapable of germination even when the perceived environmental conditions are favourable, this is also considered to be a form of dormancy. This inability to germinate has been associated with both embryo, and coat-imposed dormancy; the latter acting as a temporary physical barrier to embryo emergence, or in the prevention of adequate water imbibition or gaseous exchange into, and out of the seed. Whatever the origin of the dormancy, it is generally very precisely regulated, through environmental factors (Bewley and Black, 1982, Côme 1982 and Côme and Corbineau, 1992). There are essentially two types of dormancy, primary dormancy is established during the development of the seed whilst it is still on the mother plant, whilst secondary dormancy involves a sustained reduction in germinability which is induced when seeds are stored in unfavourable (for

germination) environmental conditions. Secondary dormancy can occur in seeds that have either displayed primary dormancy or not (Karssen, 1980, 1981a, 1981b, Bewley and Black, 1982 and Côme and Corbineau, 1992). Non-optimal temperature, light and oxygen concentrations have been shown to play important roles in the induction of secondary dormancy.

1.2 The relationship between seed metabolism and oxidative stress

1.2.1 Finding a basis for seed recalcitrance ?

It seems apparent that clues as to the basis of recalcitrance may be derived by examining the damage / repair processes occurring in such seeds during drying and storage. In this regard Hanson (1984) suggested that major emphasis should be placed on determining the basis of desiccation injury in recalcitrant seeds. Membranes, enzymes and chromosomes are cited as potential targets of injury. Unfortunately, whilst there is considerable information concerning biochemical injury in orthodox seeds (Smith and Berjack, 1995 review this topic) this area of research remains relatively unexplored in recalcitrant species. However, several factors are emerging as being of particular importance. These are dehydration injury, seed coat permeability, metabolic activity and membrane repair.

Good examples of this include the finding that *Shorea robusta* seeds can lose their viability within 10 days of ripening (Nautiyal and Purohit, 1985a), as the seed coat looses water and a decline in the physiological activity of the cotyledons and the embryo results. Similarly, permeability of *Trifolium subterraneum* was studied with

GC and the linoleic and linolenic acid content of permeable and impermeable 20-year-old seeds was measured (Flood and Sinclair, 1981). Permeable seeds exhibited significant decreases in unsaturated fatty acids, suggesting lipid peroxidation was occurring during storage resulting in a reduction of viability. Impermeable seeds were thought to exclude oxygen preventing the oxidation of fatty acids. A sequential loss of moisture from the seed coat, cotyledons and embryo of *S. robusta* lead to cellular membrane damage (Nautiyal and Prohit, 1985b) together with a reduction in proteins, carbohydrate and enzyme activity. An increase in phenols from autoxidation also occurred. Membranes damaged during storage were found to be irreparable, even after re-imbibition (Nautiyal and Prohit, 1985a, 1985b and 1985c).

Cumming and Osbourne (1978a and b) also demonstrated that in wild oat seeds, a continuous turnover of membrane lipids occurs, as opposed to a net synthesis in hydrated cells, impairment of this *status quo* results in seed death. A critical moisture content at which repair and membrane turnover may fail is thought to be the cause of viability loss upon storage in *S. robusta* seeds (Nautiyal and Prohit, 1985b and 1985c). Membrane repair involves a very complex but efficient system. Studies in mammalian and artificial systems suggest that oxidised membrane components are removed, detoxified and reinserted into the membrane.

Protein denaturation, accumulation of phenolics and changes in peroxidase activity are additional signs of biochemical damage in recalcitrant seeds (Natuyal *et al.*, 1985). The metabolic potential and its role in the precipitation of a number of these damaging events was investigated using a comparison of two species *Guilfoylia*

monosylis (recalcitrant) and *Erythrina caffra* (orthodox) by Nkang and Chandler, (1986). The recalcitrant species maintained a high enzyme activity and an increased turnover of biosynthetic pathways. The species is thus primed for seedling development as it displays no dormancy, but when placed in storage was susceptible to metabolic impairment and oxidative damage. The orthodox species however, had a much reduced metabolic potential, necessary for maintaining its dormant state and as such was less susceptible to oxidative damage. The range in moisture content in which repair mechanisms operate in orthodox seeds coincided with the range in which recalcitrant seeds start to loose most, if not all, of their viability. This is interesting because if orthodox seeds are capable of repair, does this mean that recalcitrant seeds are not ?

Farrant *et al.*, (1988) have used the seed of *Avicennia marina* to suggest a model for the behaviour of desiccation-sensitive recalcitrant seeds. When seeds are shed they are metabolically active, cell structure is compact, vacuolation is limited and mitochondria are already developed. Enhanced mitochondrial activity then occurs within the seeds with an increased rate of succinate dehydrogenase activity. Other changes follow including increased golgi activity, the accumulation of starch in plastids, increased protein synthesis, and enhanced levels of cytoplasmic and membrane-bound polysomes, making the seeds increasingly sensitive to dessication. This means that is there is a decline in the amount of water loss that can be tolerated by the cell. As a consequence, if seeds are dried too rapidly before sufficient metabolic activities have occurred they tolerate a greater degree of water loss. Farrant and co-workers (1988) argue that as molecular water has an intimate

relationship with macromolecular surfaces, ensuring the stability of membranes, then removal of this water at low moisture contents during desiccation destabilises the membrane structure (Vertuci and Farrant, 1995).

1.2.1.1 *What are the consequence of intracellular water stress ?*

Seneranta and McKersie (1986) have suggested, therefore, that there may be a heterogeneous intra-cellular and intra-organellar re-distribution of water in recalcitrant seed tissues during desiccation stress dependent upon the nature and abundance of membranes and macromolecules and their location. Desiccation stress may not be a uniform occurrence and the authors have also observed differential leakage rates from seed components upon rehydration. Leprince *et al.*, (1993) also reported differential sensitivity to desiccation in respiratory enzymes in germinating maize. With dehydration the NADPH : $NADP^+$ ratio declines rapidly resulting in a total loss of all respiratory potential. Peroxidation induced damage during desiccation could be due to monosaccharide respiratory substrates and an impaired electron transport chain, damage being hastened by a reduction in activity of free radical scavengers (Leprince *et al.*, 1990, 1992 and 1993). Work by Poulsen and Eriksen (1992) on the decline in respiratory activity of *Q. robur* axes, the increase of proline on seed dehydration and work by Salmen-Espindola *et al.*, (1994) on the decline in oxygen uptake in embryonic axes of *Araucaria angustifolia* following the loss in ability to germinate, suggest that integrated cellular metabolism is no longer possible as specific components or systems seem more susceptible to water loss than others. Leprince *et al.*, (1990, 1992 and 1993) have noted a facilitation to lipid peroxidation when this occurs.

Damage to seed cells, whether hydrated, partially dehydrated, or desiccated appears to be through lateral phase separation of membrane lipids in the plasmalemma and intracellular membrane (McKersie *et al.*, 1988). This may account for increases in solute leakage that accompany viability loss at high water contents in the axes of recalcitrant seeds (Pammenter *et al.*, 1991 and 1993).

1.2.1.2 *The possible role of free radicals in the deterioration of stored seeds*

Dehydration of the intracelluar environment interferes with metabolism and leads to the formation of free radicals due to macromolecular conformational changes, impaired intracellular transport, changes in pH / ion concentrations, and the inappropriate accumulation of various components (Senaratna and McKersie ,1986). The irreparable damage to intracellular metabolism and subsequent membrane damage was described by Senaratna and McKersie as a desiccation associated event. The involvement of gel-phase domain formation, associated with lateral phase separation of the phospholipid resulted in membrane protein displacement leading to the accumulation of free radicals and uncontrolled reactivity (Senaratna and McKersie, 1986 and McKersie *et al.*, 1988).

It is not the case that free radicals are not involved in normal metabolism, however, here they are subject to stringent control, because they are highly reactive and potentially damaging. When cells are stressed these control mechanisms are the first to fail and this may cause free radicals to be accumulated (Benson, 1990). Other types of cellular damage such as abnormal electron transport and enzyme dysfunction have a role to play. This is certainly the case in uncontrolled localised

free radical production in the absence of a phased, efficient operation of scavenging systems (Senaranta and McKersie, 1986). The possible roles for the involvement of free radicals in the loss of viability in seeds are discussed in the following section, and evidence is put forward to support these hypotheses.

1.2.1.3 *Evidence*

It has now been shown that free radicals may be implicated during the loss of desiccation in germinating seeds (Leprince *et al.*, 1990; working on maize). Leprince and co-workers saw the accumulation of an organic radical, and a significant increase in lipid peroxidation when desiccation-sensitive axis tissue was severely dried. The formation of this organic radical was ascribed to the activity of oxygen radicals generated during a desiccation-induced uncoupling of the electron transport chain. They also found that the activity of the antioxidant superoxide dismutase (SOD) also declined during this period. Another line of evidence comes from work done on the temperate recalcitrant tree species *Quercus robur* L. (pendunculate oak) by Hendry *et al.*, (1992) and Finch-Savage *et al.*, (1993). Hendry and co-workers demonstrated that free radical initiated damage occurred when the axes were dehydrated to water contents below 47 %, which also coincided with an increase in lipid peroxidation. They also showed that while hydrated axes appear to utilise α-tocopherol and ascorbic acid as antioxidants, protection against oxidative attack is conferred primarily by SOD and glutathione reductase in the cotyledons (Hendry *et al.*, 1992; Finch-Savage *et al.*, 1993). However because of the seeds recalcitrance, presumably both of these systems appear to fail at relatively high water contents.

Both of these studies, on maize and oak, have been carried out on recalcitrant material that has undergone dehydration, and provides convincing evidence that free radical mechanisms are involved in desiccation-related viability loss. They also demonstrate that because of impaired metabolism, normal cellular free radical inactivation (through scavengers or enzymes) is probably sub-optimal.

1.2.2 Oxidative stress: its origins, consequences and measurement

Normal cellular metabolism depends on the controlled movement of electrons and the involvement of molecular oxygen in respiration and other oxidative processes. Under conditions of environmental stress i.e. physical injury, ageing and disease control (in the form of tight metabolic coupling and cellular compartmentalisation) is removed and highly reactive, destructive free radical species are produced (usually represented as $R^{\bullet}$). These chemically reactive molecules have an unpaired electron and as such readily react with other molecules initiating a cascade (chain) reaction, thus resulting in a rapid accumulation of free radical species.

1.2.2.1 *The oxygen paradox*

Protection against free radical injury is therefore essential, particularly within an aerobic cellular environment as very often free radicals are produced as oxyradicals, one of the most damaging of all radicals produced by the cell. These along with a range of intermediate breakdown products of proteins, lipids, carbohydrates and nucleic acids formed from the stepwise electron transfer in primary reactions serve attack the cell as a whole. Often the free radicals formed through initial damage to the cell go on to cause further damage. Because of their extreme reactivity it is

difficult to determine whether they are a direct cause of injury or a consequence of preceding damage, or both.

1.2.2.2 *The chemistry of free radical formation and molecular oxygen*

1.2.2.2.1 *Free radical formation and oxyradicals*

Free radicals are toxic because of their highly reactive chemical properties, but *why* are they so reactive ? When formed they do not exhibit normal bonding behaviour where electrons are paired, possess charge and spin and thus confer bond stability by each electron pairing in opposite spins (i) (that is the electrons are shared unequally between component molecules).

Instead, free radicals are formed when molecular bonds between two electrons are split homolytically rather than heterolytically resulting in the electrons being shared equally, resulting in free radical species with unpaired electrons (ii).

(i) $w : x \rightarrow w: + x$ or $w + x:$

(ii) $w : x \rightarrow w^{\bullet} + x^{\bullet}$

Because of the need of these free radicals ($w^{\bullet} + x^{\bullet}$) to pair with the electrons of neighbouring molecules they are highly reactive. For example a hydroxyl radical has a half life of 10^{-9} seconds.

Atmospheric oxygen can be toxic as molecular oxygen is a biradical, possessing two unpaired electrons of parallel spin. These unpaired electrons when bonding with

another chemical must enter a bond share, or convert its spin states to produce an electron with an antiparallel spin. This conversion leads to the production of highly reactive free radicals such as the superoxide radical ($O_2^{\bullet}$-). This radical is involved in a group of reactions jointly known as the Haber-Weiss and Fenton reactions (Benson, 1990).

1.2.2.2.2 *The Haber-Weiss and Fenton reactions*

The reaction between $O_2^{\bullet}$- and H_2O_2 as proposed by Haber is:

$$H^+ + H_2O_2 + O_2^{\bullet}- \rightarrow O_2 + H_2O + {}^{\bullet}OH$$

The reaction produces a hydroxyl radical ($^{\bullet}OH$), a highly reactive molecule in biological chemistry.

Fenton demonstrated that the above reaction is a two step process. Firstly, the superoxide radical then reduces the ferric ion.

$$Fe^{3+} + O_2^{\bullet}- \rightarrow Fe^{2+} + O_2$$

The ferric ion then reacts with H_2O_2 to form a hydroxyl radical.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$$

Controversy surrounds the Fenton reaction mechanism *in vivo*, as it is thought that the hydroxyl radical is not alone in systems containing $Fe^{3+,2+}$, $O_2^{\bullet}$- and H_2O_2 (Benson, 1990).

1.2.2.2.3 *Propagation and termination reactions*

The fate of the free radical is an important consideration when evaluating their toxicity, the longer lived they are, the greater the level of cellular exposure. Free radical stability can vary considerably depending on their composition and interaction within different molecular environments. The length of the half life of free radicals is dependent upon when termination of the reaction occurs, this is usually when two free radicals combine to form a molecule with paired electrons, and hence confer bond stability.

Free radicals are generated by several mechanisms often in cascade, because of this self propagation and their high reactivity they are in no doubt toxic to living organisms. These mechanisms include:

1. Addition reactions to double bonds

$$w^{\bullet} + x{=}y \rightarrow w\text{-}x + y^{\bullet}$$

2. Cleavage

$$^{\bullet}w\text{-}x\text{-}y \rightarrow w^{\bullet} + x{=}y^{\bullet}$$

3. Electron transfer

$$w^{\bullet} + x^{-} \rightarrow x^{\bullet} + y^{-}$$

The cascades are terminated by a random interaction that results in a termination step. Slater (1984) categorised these steps as:

1. Dimerization

$$w^{\bullet} + x^{\bullet} \rightarrow w\text{-}x$$

2. Disproportionation

$$w^{\bullet} + x\text{-}y\text{-}z \rightarrow w\text{-}x + y{=}z$$

3. Electron transfer

$$w^{\bullet} + x \rightarrow w^{+} + x^{-} \text{ or } w^{-} + x^{-}$$

In biological systems termination can also occur by the intervention of protective mechanisms such as free radical scavenging reactions.

1.2.2.2.4 *Effects of free radical reactions within biological systems*

Harman (1956) suggested that the formation of activated oxygen species is the *single* underlying cause of ageing, which is modified by genetic and environmental factors, and involves random and deleterious damage by free radical reactions formed during mammalian anaerobic metabolism. The application of this theory to plants and particularly seeds is, however, far from resolved: for example it has been reported that recalcitrant seeds show an increase in free radical intensity upon desiccation (Hendry *et al.*, 1992). However, Magill *et al.*, (1994) have shown that other forms of stress do not necessarily lead to increases in signal intensity (e.g. when papaya seed is wet chilled).

Plants and higher vertebrates, possess a complex array of oxygen scavenging systems (tocopherols) and enzymes (peroxidases, catalases and superoxide dismutases) whose purpose it is to scavenge activated oxygen species (Priestly, 1986, Benson, 1990 and Winston, 1990). Such defence mechanisms, complemented by compartmentalisation are, not surprisingly, found predominantly in organelles where respiratory metabolic turnover is high, such as the mitocohdrion. It is now recognised that all aerobic organisms have evolved these mechanisms, to help prevent excessive oxidative damage. However, if this normally tight metabolism is challenged by stress, ageing or disease, there is an increased potential for free radical production. If this increase is higher than the cells normal metabolism would encounter, there is a serious, and possibly critical, challenge to the protective antioxidant mechanisms. If the challenge exceeds these protective mechanisms oxidative damage may ensue (Benson, 1990).

The importance of such enzymes has already been noted in some primates where life span potential is strongly correlated with oxygen consumption / metabolic rate and SOD activity (Cutler, 1984). Moreover, the observation that a mutation in the SOD gene results in a progression of a fatal neurological disease in humans strongly suggests that such regulatory genes are determinants of longevity, acting at the molecular level.

1.2.2.2.5 *Why is elemental oxygen so important for seed biology ?*

Apart from its abundance, oxygen has two outstanding characteristics. It is, or can be made highly reactive, and is as such, unsurprisingly, a component of numerous

biological reactions. From the last few sections it is clear that throughout much of the literature, free radicals and lipid peroxidation events (which are undoubtedly mediated by free radicals) are the molecular events through which environmental stresses can exercise their damaging effects. The application of this hypothesis to seeds stemmed from research carried out by Harrington (1973), Stewart and Bewley (1980), Bewley (1986) and Wilson and McDonald (1986). It showed that aged seeds displayed characteristic changes in their polyunsaturated fatty acids and that the presence of oxygen exacerbated this response.

Breakdown products of free radical mediated lipid peroxidation, are both cyto- and geno-toxic. The free radicals and hyperoxides formed during lipid peroxidation can decompose to form secondary breakdown products (Gutteridge and Halliwell, 1990) such as monohydroxyphenols. Such secondary products of lipid peroxidation, are unstable and can decompose to produce aldehydes and more free radicals.

These breakdown products interact with $OH^{\cdot}$ or $H^{\cdot}$, producing low molecular weight hydrocarbons such as ethane, propane, pentane and butane. Alternatively, monohydroperoxides can interact with molecular oxygen, to form a range of peroxides which can decompose into many different volatile breakdown products (Benson, 1990). Malondialdehyde (MDA), for example, is one of the more investigated secondary breakdown products of lipid peroxidation, and has been found to be cytotoxic and mutagenic (Frankel, 1987). MDA has been found in stressed, and ageing animal tissue, and causes damage to DNA, proteins and enzymes by interacting with them to form a range of fluorescent compounds (Schiff's

bases), collectively named lipofuscins or 'age pigments' (Frankel, 1987). MDA has yet to be detected in seed tissue, stressed or unstressed. However, similar fluorescent compounds have been detected in micropropagated *Vitis viniflora* L. plants as they aged in culture (Benson and Roubelakis-Angelakis, 1994).

A good example of the mutagenicity of MDA has been shown in microbial systems using *Escherichia coli*. Akasaka and Yonei (1985) found that the mutation frequency in *E. coli* cells to streptomycin resistance increased greatly when the cells were incubated in a reaction mixture of microsomal lipid peroxidation products. Such that the mutation frequencies were determined by the extent of lipid peroxidation that had taken place. This clearly demonstrates that free radical mediated lipid peroxidation, may have crucial secondary effects on the cells molecular activity.

Protective mechanisms that could scavenge the peroxidatively produced free radicals and peroxide have evolved within seed cells to keep these deleterious compounds to a minimum. Such mechanisms involve several free radical and peroxide scavenging enzymes such as SOD, catalase, peroxidase and ascorbate peroxidase (Harrington, 1973, Halmer and Bewley, 1984 and Bowler *et al.*, 1992). Thus the corollary of the above suggests that the longevity of seeds under hydrated storage conditions, would be shorter if the activity of such enzymes was inhibited (dry seed reactions are non-enzymic).

The dependency upon oxygen relates to the fact that oxygen can exist in many forms O_2, O_3, O_{12}, $O_2^{\cdot-}$, and, of course, as the hydroxyl radical ($HO^{\cdot}$). It is the terminal

electron acceptor in electron transport chains, during plant respiration and is evolved during photosynthesis. Consequently, plants are totally dependent on an adequate, but not necessarily constant, supply of oxygen. Another consequence of this, is that the product of respiration, water, is chemically inert, unlike products of anaerobic respiration. Oxygen, therefore, offers considerable advantages over other electron acceptors.

In oxidase reactions di-oxygen (O_2) is involved directly in the acceptance of one or more, additional electrons, the maximum being four electron reduction of di-oxygen to water, as in cytochrome C oxidase during aerobic respiration:

$$4H^+ + O_2 \xrightarrow{4e^- + \text{Cytochrome C}} 2H_2O$$

However, many biological reactions involve less than four electrons, increasing the opportunity for the formation of more reactive oxygen free radical species, which can cause damage through lipid peroxidation.

The immediate problem with considering oxygen as having both sub-lethal and lethal effects is to expose the uncertainty of what is cause and effect. Some years ago Halliwell and Gutteridge (1984) warned of the dangers of considering activated oxygen necessarily as the cause of tissue damage, or death, whereas it could without further enquiry, also arise as the result of death or disease. That is lipid peroxidation may be the *consequence* of earlier tissue damage and not itself the cause.

1.2.3 The measurement of oxidative stress in seed germplasm

Quite apart from the many techniques which measure the consequences of free radical production such as lipid peroxidation, several methods aim to measure the extremely transient radicals themselves. These techniques depend upon detecting the first, or early products of radical-mediated reactions *in vitro*, under strictly controlled conditions. For example, by direct detection by electron paramagnetic resonance (EPR) spectroscopy, formerly known as electron spin resonance (ESR) spectroscopy.

1.2.3.1 *Direct measurement of free radicals*

EPR spectroscopy is a technique which can detect free radicals (paramagnetic species) directly, and has been used extensively to investigate irradiated food (Knowles *et al.*, 1976), but has not been used widely in relation to seed germplasm. The technique relies upon the principle that electrons possess both spin and charge, and therefore create their own magnetic fields. If an unpaired electron is exposed to a constant form of electromagnetic radiation, the electron can undergo spin reversal (resonance). The frequency of electromagnetic radiation at which this occurs, is a function of the paramagnetic species, and the applied magnetic field. In practice the biological sample is subjected to a constant form of electromagnetic radiation (usually within the microwave frequency), and the applied field is varied until resonance occurs, showing the presence of a paramagnetic species (reviewed in Assenheim, 1966, Knowles *et al.*, 1976 and Benson 1990). Such methods have been used to examine the free radical processes involved in aged seed material, in recent reports by Hendry *et al.*, (1992), Magill *et al.*, (1994), Finch-Savage *et al.*, (1994) and Goodman *et al.*, (1995).

Because many free radicals are highly reactive, it is not always possible to detect them using EPR alone, and a technique known as `spin trapping` is also incorporated (Williams and Wilson, 1975). In this method, diamagnetic organic molecules (spin-traps) react with free radicals to produce a longer-lived, stable radical adduct which can then be subsequently detected.

1.2.3.2 *Indirect measurement of intermediates and products of free radical reactions*

Several compounds can be formed during the propagation, termination and stabilisation events of free radical formation. The secondary reactions of oxidative injury can also produce a range of intermediates. Free radicals reactions occur throughout the entire cellular metabolism, and hence they or their intermediates / products have the potential to damage a range of cellular components, this can result in a plethora of breakdown products being formed.

Wilson and McDonald (1986) reviewed the various techniques used on seed material in determining oxidative damage. They looked at membrane characteristics such as lipid / phospholipid content, bond saturation, the release of free fatty acids, and the production of lipid peroxides and their associated breakdown products. They also conducted tests on membrane phase changes and alterations in permeability characteristics using differential scanning calorimetry and solute leakage measurements.

Gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) together with more routine UV / visible spectroscopy,

fluorimetry and thin-layer chromatography (TLC) measurements were conducted to detect lipid peroxidation and associated membrane damage. Harman and Mattick (1976) and Wilson and McDonald (1986) quantified the linoleic and linolenic acid fractions in aged seeds and alterations in phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine and determined the changes in the saturations of fatty acids using this instrumentation. Senaratna *et al.*, (1985 and 1988) found GC and TLC the most sensitive methods to measure changes in lipid bond saturation by quantifying free fatty acid release from aged soybean seeds.

Choudhuri and Basu (1988) measured lipid peroxidation in onion seeds using an MDA spectrophotometric assay. MDA lipid peroxidation breakdown products can cross-link with proteins and DNA and the resulting products can be detected by their fluorescence. Lipid peroxidation in senescing cotyledons of *Phaseolus vulgaris* L. has been detected using fluorimetry (Pauls and Thompson, 1984). Arai *et al.*, 1967 and 1970; Spencer *et al.*, 1973 detected the breakdown products of lipid peroxidases, aldehydes and acetyl in seed oil extracts, and whole seeds from a wide range of species subjected to different ageing treatments. Many of these have been previously identified as the toxic breakdown products of free radical-mediated injury in mammalian tissues.

1.2.3.3 *Measurement of antioxidant status*

The measurement of antioxidant status combined with the examination of free radical reactions can also be used to monitor free radical activity. As a number of environmental and developmental factors can influence levels of antioxidants in

tissues it is useful to conduct such studies in conjunction with assays which measure free radical activity *per se.*

The majority of the assay procedures used to investigate antioxidant status involve substrate / enzyme reactions detectable by UV / visible spectroscopy. Peroxidases can be assayed using a technique described by Murphy and Huerta (1990), where guiacol is oxidised to form an orange pigment which absorbs maximally at 470 nm. Catalase concentration can be determined via the reduction of hydrogen peroxide, which can be measured at the UV wavelength of 240 nm.

Soybeans were aged for five years at room temperature and their relative antioxidant activity was determined by their ability to inhibit Fe^{2+} catalysed oxidation of linoleic acid in seed extracts Senaratna *et al.*, (1985). UV / visible spectroscopy has been used to measure ascorbate and dehydroascorbate levels (Senaratna *et al.*, 1985). In addition, Paul and Thompson, 1984 monitored levels of reduced glutathione (GSH), oxidised glutathione (GSS), SOD and catalase in senescing cotyledons of *P. vulgaris.*

1.3 Objectives of this study

To assess the nature of the oxidative stress response to drying in seeds which are desiccation-intolerant, using physiological, biochemical and biophysical parameters (Chapters 3 and 4).

To relate these findings to a wider range of material in order to develop an understanding of desiccation-intolerance with respect to drying sensitivity and oxidative stress. Recalcitrant species of varying desiccation sensitivity are investigated (Chapter 5), to assess the possibility that oxidative stress responses could be used as a diagnostic indicator for seed storage category.

To determine the role of oxidative stress in seed survival. The possibility that oxidative stress may be used as an indication of seed germinability in recalcitrant and orthodox seeds is investigated through desiccation and storage of both papaya and horse chestnut seeds (Chapter 6).

Finally, to acertain if an interaction exists between oxidative stress, germinability, dormancy and relative desiccation sensitivity in papaya seeds (Chapter 7). This section also describes a model for dormancy release in papaya seeds, and discusses the possible misclassification of this species storage categorisation.

CHAPTER 2

General Materials and Methods

Chapter 2: General materials and methods

Outlay of Chapter

Chapter 2: General Materials and Methods

Details of the materials used and general techniques employed are provided within this chapter. Additional details relating to specific aspects of experimental design are given in relevant chapters.

2.1 Seed procurement and germination

2.1.1 Seed procurement and harvesting

2.1.1.1 *Plant material*

Details of each seed batch used during this study are given in Table 2.1. For three of the species investigated more than one seedlot was used. The term 'batch' (B) describes seeds collected from different plants, or from the same plant in different seasons. Whilst the term 'seedlot' (L) describes seeds collected from the same plant at different times in the same season. In this report seeds will be referred to by their common names, if known.

The conditions and duration of seed storage after collection or receipt, and before their investigative use are detailed in Table 2.2. Seeds were used at the end of the time given, following storage under the specified conditions, unless otherwise stated in the relevant chapters. Gifts of seed were made by Dr P Haley (cacao), Reading University and Dr A Maderious (Brazilian pine), EMBRAPA-Cenargen, Brazil.

Table 2.1
Origin of each batch of seeds.

Species[1]	Batch[2]	Common name	Source[3]	Origin
Aesculus hippocastanum	**B1**	**horse chestnut**	**C**	**Chailey, East Sussex, UK**
Aesculus hippocastanum	**B2**	**horse chestnut**	**C**	**Chailey, East Sussex, UK**
Aesculus hippocastanum	**B3: L1-L4**	**horse chestnut**	**C**	**Wakehurst Place, West Sussex, UK**
Araucaria angustifolia		**Brazilian pine**	**G**	**Cenargen, Brazil**
Azadirachta indica		**neem**	**PC**	**India**
Carica papaya	**B1**	**pawpaw**	**PC**	**Costa Rica**
Carica papaya	**B2**	**pawpaw**	**PC**	**Costa Rica**
Carica papaya	**B3**	**pawpaw**	**PC**	**Jamaica**
Castanea sativa		**sweet chestnut**	**PC**	**France**
Quercus robur		**pedunculate oak**	**C**	**Wakehurst Place, West Sussex, UK**
Quercus rubra		**red oak**	**PC**	**Holland**
Theobroma cacao		**cacao**	**G**	**Eary Gate, Reading University, UK**

[1] After commonly recognised naming authority
[2] Batch (B), Seedlot (L)
[3] Collected (C), Gift (G), Purchased commercially (PC)

Table 2.2
Conditions of storage for each batch of seeds prior to use.

Species	Batch[1]	Date of receipt	Storage before use[2]		
			Conditions	Container	Time (weeks)
Aesculus hippocastanum	B1	10 / 92	16 °C	pb	< 1
Aesculus hippocastanum	B2	10 / 93	16 °C	pb	4
Aesculus hippocastanum	B3: L1-L4	10 / 94	16 °C	pb	2
Araucaria angustifolia		05 / 94	2 °C	pb	< 1
Azadirachta indica		09 / 95	16 °C	cb	2
Carica papaya	B1	01 / 94	15 °C / 15% RH	fb	6
Carica papaya	B2		15 °C / 15% RH	fb	
Carica papaya	B3		15 °C / 15% RH	fb	
Castanea sativa		11 / 93	2 °C	pb	1
Quercus robur		10 / 92	2 °C	pb	2
Quercus rubra		11 / 93	2 °C	pb	1
Theobroma cacao		06 / 95	16 °C	pb	< 1

[1] Batch (B); Seedlot (L).
[2] Cotton bag (cb); dried to stated % RH before being heat sealed within the foil bag (fb); loosely tied black polythene bag (pb).

2.1.1.2 *Seed procurement and harvesting*

Seeds from seven species of plant were used throughout this investigation; *Aesculus hippocastanum* L. (horse chestnut), *Quercus rubra* L. (red oak), *Quercus robur* L. (pendunculate oak), *Carica papaya* L. (papaya), *Theobroma cacao* L. (cocoa), *Araucaria angustifolia* L. (Brazilian pine), and *Azadirachta indica* L. (neem). Acquisition of the seeds varied considerably depending upon species:

- Horse chestnut - were harvested in October 1993 and 1994 from a stand of more than 25 trees at Chailey, East Sussex (ordinance survey ref. TQ / 333722). The 1995 collection from Chailey was unsuccessful, frost early in the season had severely reduced seed numbers. In an attempt to enhance the collection, two horse chestnut trees at Wakehurst Place, West Sussex (ordinance survey ref. TQ / 342313) were also used. Seeds were stored for short periods (see Table 2.1) of time, hydrated in the dark at 16 °C in folded over black polythene bags until needed.

- Pedunculate oak and red oak - seeds were purchased commercially (Forestry Commission, Alice Holt Lodge, Surrey) in November 1993 and 1994. Seeds were thoroughly mixed and stored for short periods (see Table 2.1) of time, hydrated in the dark at 2 °C in folded over black polythene bags until needed.

- Papaya (var. solo) - three commercial batches of Papaya fruits were used. Batches one and two were of Costa Rican origin and were purchased from Henry Mears of

Lewes, East Sussex in November 1993 and April 1995 respectively. The third batch was purchased from J. Sainsburys Ltd, in East Grinstead, West Sussex in February 1996 and were of Hawaiian origin.

- Cacao - seed pods were kindly supplied courtesy of Dr Paul Hadely from a collection maintained at the Department of Agriculture, University of Reading, U.K. The pods were stored at 16 °C in the light until needed, at this point the embryos were extracted and used immediately.

- Brazilian pine - seeds were supplied by Dr Antonio Madeires (EMBRAPA-Cenargen, Brazil). Seeds were stored hydrated upon arrival in folded over clear polythene bags at 2 °C, until needed.

- Neem - seeds were supplied commercially by Green Gold International (Ludhiana, India). Seeds were harvested in August 1995 and received at Wakehurst Place on the 7th September 1995. Immediately upon arrival they were transferred from the cotton bags used for transit to inflated polythene bags, and then stored at 16 °C until needed.

In all of the above cases, when seeds were stored in polythene bags, the bags were loosely tied, and were ventilated regularly. The contents were always thoroughly mixed before seeds were withdrawn for experimentation.

2.1.2 Germination treatments - growth conditions and media

For horse chestnut seeds two replicates of 15 seeds per treatment were sown on 1% agar in distilled water (Lucas Meyer) in clear plastic boxes (173 x 115 x 60 mm), which were wrapped in aluminium foil (to exclude light) and placed at 35 °C (Pritchard and Steadman, pers. com.). The same method of seed germination was employed for oak seeds except that the foil-wrapped plastic sandwich boxes, were placed at 16 °C.

Papaya seeds upon extraction from the fruit, were carefully blotted using absorbent paper to remove the fleshy sarcotesta, and then cleaned by briefly rinsing twice in distilled water. For germination testing, two replicates of 25 seeds per treatment were sown on 1 % agar in distilled water in 9 cm Petri dishes placed initially at 26 °C with a 12h day-length (photon flux density c. 150 W m^{-2} s^{-1} from warm white light fluorescent tubes). Alternative temperature regimes are described fully in Chapter 7.

Neem and cacao seeds were germinated on 1% agar in distilled water, in 9 cm diameter plastic Petri dishes, at 26 °C with a 12h day-length (photon flux density c. 150 W m^{-2} s^{-1} from warm white light fluorescent tubes). All incubation was carried out in the light, 15 seeds were used for each germination treatment. To reduce the chance of fungal and bacterial cross-contamination, seeds were spaced equally on the plates, rotten seeds were discarded.

Brazilian pine seeds were germinated on 1% agar in distilled water in clear plastic boxes at 16 °C. Fifteen seeds were used per treatment, and boxes were wrapped in aluminium foil to exclude the light.

The specifics for germination of each species varied considerably, and care was taken to carefully characterise the morphology of germination for each species.

2.1.3 Germination measurement and assessment

In horse chestnut and oak seeds germination was recorded when radical extension was greater than or equal to 10 mm (see Chapters 4 and 6). Papaya seed germination was generally recorded when radicle extension was greater than or equal to 5 mm (see Chapter 7). Neem seed germination (radicle emergence) was assessed at regular intervals, and a positive score given when radicle emergence exceeded more than 1 mm. Brazilian pine and cocoa germination was deemed positive when radical extension was greater than or equal to 20 or 10 mm respectively.

2.2 Desiccation treatments and moisture content determination

2.2.1 Desiccation procedure and measurement of relative humidity

Papaya and neem seeds were initially measured for % equilibrium relative humidity (eRH) using an adapted version of a Michell series 4020 (Michell Instruments, Cambridge, U.K.) precision dewpoint hygrometer. Later in the study a Rotronic Hygroskop DT (Rotronic Instruments U.K. Limited, Horley, U.K.) precision

hygrometer was also used for relative humidity measurements, particularly when monitoring whole seed humidity values during desiccation experiments.

Generally whole seeds were dried as a mono-layer on slatted trays in a dry-room maintained at 15 ± 1 °C and 15 - 20 % relative humidity, with an air flow of 0.5 m s^{-1}. If dried and stored they were hermetically sealed in laminated foil bags and held at 15 °C until needed. Seeds were removed regularly to determine moisture content and equilibrium Relative Humidity (eRH) values using two replicates of five seeds.

Desiccation periods varied with species; up to 80 h for horse chestnut and c. 4 weeks for Brazilian pine. Drying room conditions were checked regularly with the use of a Rollog Agent HT1-A monitoring unit (Rotronic Instruments U.K. Limited, Horley, U.K.), an example output from this device is shown in Appendix III. Following desiccation seeds were generally placed directly on 1 % agar in distilled water at the relevant germination temperature to rehydrate, as part of the germination treatment.

2.2.2 Moisture content determination and rehydration treatments

All moisture contents were determined gravimetrically, using either a five- or seven-place balance (Sartorius Instruments, Göttingen, Germany) following drying in an oven at 103 °C for 17 ± 1 h, in accordance with the International Seed Testing Association guidelines regarding non-oily seeds (ISTA, 1985) All moisture contents are expressed on a f. wt. basis.

For horse chestnut seven individual seeds were used for moisture content determination of both the embryonic axes and remaining seed tissue (testa and cotyledons combined). For papaya five individual seed moisture content determinations were made per treatment; generally at the component tissue (endosperm, testa and embryo) level. Equilibrium relative humidities were also measured for papaya seed using the Michell series S-4020 dewpoint hydrometer. Individual seeds for each measurement were equilibrated over a 1 h period at 21 °C. In neem seed, moisture contents were determined on 5 individual seeds per treatment, again equilibrium relative humidities were also measured for neem, however, using the Rotronic Hygroskop DT hydrometer. Samples of 5 seeds for each measurement were equilibrated over a 1 h period at 21 °C. Brazilian pine and cacao moisture contents were determined at a seed component level; female gametophyte, embryo, scale-bract, respectively and cotyledons and embryo respectively, as described above. Five seeds were used for each determination.

2.3 Free radicals, determination and measurement

2.3.1 Electron paramagnetic resonance spectroscopy

2.3.1.1 *Tissue preparation and spectral parameters used*

Biological samples containing 'free' water (unbound; type III) can be problematic for EPR spectrometry as it absorbes microwaves (see Knowles *et* al., 1976 and Chapter 3). There are three ways in which type III water can be removed, or at least

'masked', during sample preparation: freeze-drying, the surviving-tissue technique and by rapid-freezing.

In freeze-drying, water is removed from the tissue by lyophilization and the sample can then be examined in a standard EPR tube. The method is reported to have good sensitivity (Knowles *et al.*, 1976), but it is uncertain what proportion of the *in vivo* free radicals survive the freeze-drying process. It is also possible that artifactual free radicals are generated during the freeze-drying process. For these reasons, the freeze-drying technique was not used.

The surviving-tissue technique approaches most closely to that of the *in vivo* situation. The tissues to be studied are removed as quickly as possible from the sample and stored on ice. Tissue slices are then cut as required (c. 0.5 mm thick) and examined in a tissue cell, or in aqueous suspension in a flat sample cell (Knowles *et al.*, 1976). The major disadvantage of this technique is that it suffers from low sensitivity, because the relatively high water contents present in the samples.

Rapid-freezing overcomes the difficulty of high water contents by using samples that are rapidly frozen in liquid nitrogen. The samples are then examined in the spectrometer at this temperature.

Excised seed tissues from stored and / or desiccated treatments were initially frozen in liquid nitrogen. Frozen samples, upon analysis, were directly transferred to a pre-cooled silvered, vacuumed quartz finger dewar containing liquid nitrogen. This was

placed in the ER4103TM resonant cavity of a Bruker ESP300E X-band spectrometer (Bruker Spectrospin, Coventry, U.K.). Free radical signal intensity was measured for each treatment typically at -196 °C (77 K), using a microwave power of 12.6 μW, a modulation amplitude of 0.4 mT, a modulation frequency of 100 kHz and a sweep width of 5 mT. In some cases these parameters were changed slightly depending upon the signal obtained from the sample, these changes are highlighted in the relevant chapters. Care was always taken to position the dewar so that the specimens were located in the same region of the spectrometer cavity.

2.4 Lipid peroxidation product extraction and determination

Indirect measurement of oxidative activity involves the detection of intermediates and products of free radical derived reactions. These compounds are formed as a consequence of the propagation, termination and stabilisation and the secondary reactions of oxidative injury. Because free radicals can attack all cellular components, many different types of breakdown product can result. However, the most common method of indirect detection involves the measurement of free radical damage to membranes and their components, particularly lipids. The chemistry of the reactions which initiate and cause this type of damage, and an general introduction to how they can be measured indirectly are described in section 1.2.2.2.

2.4.1 Preparation of extraction buffer and method of seed extraction

Biological assays were used for all indirect biochemical determinations. This required seed material to be extracted into a clear non-particulate buffered solution. All extractions were maintained in 0.05 M phosphate buffer, which consisted of: 0.05 M phosphate buffer with 3.36 mg of EDTA, 0.74 mg of KCl and 1.47 mg $CaCl_2$ per 100 ml. The addition was of mono- (potassium di-hydrogen orthophosphate) to di-basic potassium phosphate (di-potassium hydrogen orthophosphate) at a pH of 7.8.

Seed material was extracted into cold buffer to minimise metabolic and enzymatic activity within the seed material. The general procedure is outlined below:

1. Seed tissues were transported and stored in liquid nitrogen within Nalgene ampules (Nalgene Company, Rochester, U.S.A.). After the excess nitrogen had evaporated off upon removal from the storage dewar, the seed components were weighed, and the fresh weight of the sample determined. The material was then quickly re-immersed in liquid nitrogen, in order to make the material slightly brittle.

2. Using a pre-chilled pestle and mortar kept on ice, frozen seed components were ground until a homogeneous 'paste' was formed. This process was time independent for both species and tissue type, but constant time periods were used for particular seed components, from individual species.

3. Phosphate extraction buffer (pH 7.8) was then added to a pre-chilled centrifuge tube on ice, and 2.5 ml of ground seed `paste` was incorporated, using an alcohol dipped, grease-free spatula. The cold temperature tended to bind the `paste` together, allowing it to drop easily into the buffer, without sticking to the sides of the centrifuge tube.

4. Each centrifuge tube was then thoroughly mixed for exactly 30 seconds, and then immediately returned to ice. After this they were centrifuged for 10 mins at 4,000 rpm, using a Sanyo-MSE chillspin (Sanyo-MSE, Cambridge, U.K.) set to -5 °C. The supernatant was then removed (it contained soluble protein and lipid peroxidation products), and added to a pre-chilled eppendorf on ice. The sedimented pellet was then kept in cold store at -20 °C as a back-up measure.

5. The eppendorf containing the supernatant was then placed in a chilled microfuge (Sanyo MSE, Cambridge, U.K.), within a cold room (0 to -5 °C), and centrifuged for a further 10 mins at 13,000 rpm. The resulting clear supernatant was again placed into a clean Eppendorf on ice, and this was re-centrifuged for a further 10 mins at 13,000 rpm, again in the cold room. The clear supernatant was removed and placed in a clean, pre-chilled cryo-vial on ice, which was frozen down and held in liquid nitrogen until needed for assay. When the sample was required it was thawed on ice. The thawing process was speeded up by gentle centrifugation using the chill spin when appropriate.

2.4.2 Preparation of standards and acids

Preparation of standards was achieved through serial dilution of a stock MDA solution. A 10 mM stock solution (99 % MDA) was serially diluted to the appropriate amounts. (i.e. 2 μM MDA was equal to 20 μl stock solution, 4 μM equal to 40 μl stock, all made up to 100 ml).

The trichloroacetic (TCA) and thiobarbituric (TBA) acids needed for the colourimetric determination of thiobarbituric reactive substances, were made up using a 20% (w / v) TCA solution, and a 0.5% TBA solution. The TBA solution was then added carefully to the TCA solution, and made up to 500 ml when cooled. TCA and TBA solutions were usually made fresh, or used only if they had been stored for 1 to 3 d.

2.4.3 Fluorimetric determination of thiobarbituric acid reactive substances

A fluorimetric determination of thiobarbituric acid reactive substances (TBARS) was made following the procedure of Fraga *et al.*, (1988) which relies upon the following reagents:

- MDA standards (see section 2.4.2 for preparation)
- 3 % (w / v) sodium dodecyl sulphate
- 0.1 N HCl
- 10 % (w / v) phosphotungstic acid
- 0.7 % (w / v) 2-thiobarbuturic acid (made on the same day as the assay)

Once the assay is initiated, the analysis had to be carried out as rapidly as possible to ensure that chemical decomposition was kept to a minimum. To ensure parity between samples, each was treated identically. Standards were always prepared at the same time as the tissue samples. The methodology was simple systematic addition of the reagents to pre-labelled centrifuge tubes, in the following order:

1. 0.5 ml of 'liquid' sample (or tissue equivalent; e.g. 0.4 ml of distilled water with 0.1g accurately weighed cellular material)
2. 0.5 ml of 3 % (w / v) of SDS, which is then vortexed thoroughly (the vortex time was standardised for each sample)
3. 2 ml of 0.1 N HCl is carefully added along with 0.3 ml of 10 % (w / v) phosphotungstic acid and 1 ml of 0.7 % (w / v) TBA.

The tubes were then transferred to a boiling water bath, located in a fume hood, and boiled for exactly 30 min. Loss of vapour from the tubes is likely during the boiling process, and may affect assay volume, and hence assay conditions and/or accuracy. To prevent this loss of vapour the tubes were covered by acid-rinsed, clean glass marbles.

After 30 min, the tubes were removed, and transferred immediately to an ice bucket for 10 min to stop the reaction.

1-butanol (5 ml) was then added, the tubes inverted 10 times in order to transfer the coloured aldehydic TBA complexes to the solvent layer. Each tube was treated in

exactly the same way (same mixing time, number of inversions). Only some of the colour-complex was transferred to the butanol layer, the rest was retained in the liquid sample extraction layer; this is quite normal.

The base of each tube was then wiped to remove excess moisture after removal from the ice, and the tubes spun for 10 min at 3,500 G. The coloured top solvent layer was removed using a Pasteur pipette, to a clean, pre-labelled glass test tube. By this stage the top layer contained a homogeneous extract of pigmented solution. Care was taken to ensure the transferred samples were visibly clear, as a quality control step. Any cellular debris will obviously interfere with the assay. The fluorimeter was set at an excitation of 515 nm and an emission of 555 nm, and the samples measured, using a sample reagent blank to zero the samples.

2.4.3.1 *Expression of results*

2.4.3.1.1 *Standard curve*

A standard curve was constructed for MDA and concentrations of thiobarbituric reactive substances determined from this. The plot was of fluorescence against concentration (see Appendix I). The results were then be expressed on a f. wt. basis, later corrected to a d. wt. basis.

2.4.3.1.2 *Terminology*

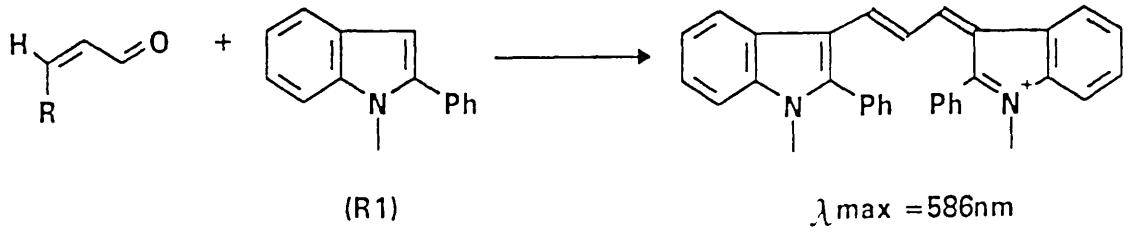
When this simple assay procedure was first developed it was thought that it was specific for MDA, however, analytical studies using HPLC and GC-MS have shown that TBA reacts with a wide range of lipid peroxidation products (Esterbauer and

Cheesman, 1990). A more acceptable and recommended method of expression of data from these assays is to express the results as thiobarbituric acid reactive substances (TBARS). These were quantified as MDA equivalents (as MDA standards were used during experimental determinations).

2.5 Determination of lipid peroxidation using the LPO-586 assay kit

MDA and 4-hydroxyalkenals, such as 4-hydroxy-2(E)-nonenal (4-HNE), are important decomposition products of peroxidation reactions. They are derived from polyunsaturated fatty acids and related esters, and their measurement provides a convenient index of lipid peroxidation (Esterbauer and Cheeseman, 1990). The classical determination of MDA by the means of thiobarbituric acids (see last section) is affected by factors such as interference, and reproducibility can be limited in some (mainly animal) tissue types (Janero, 1990).

The LPO-586 method (Bioxytech, S.A., Bonneuil/Marne, France) takes advantage of a chromogenic reagent 'R1' which reacts with MDA and 4-HNE at 45° C. Condensation of one molecule of either MDA or 4-HNE with 2 molecules of reagent 'R1' yields a stable chromophore with maximal absorbance at the 586 nm wavelength.



The LPO-586 method can be used for specific assays of MDA and 4-HNE in a single aqueous sample. Because of its simplicity, it is the method of choice for the measurement of lipid peroxidation in a large series of samples. An adaptation of the LPO-586 method enabled us to measure specifically the amount of MDA which is present in a sample containing 4-HNE (see section 2.5.5). Specific measurement of these two parameters in a given sample can be used to evaluate the possible contribution of enzyme pathways (e.g. biosynthesis of thromboxane) in the production of MDA.

Each assay kit contained two reagents 'R1' and 'R2', and two standards 'S1' and 'S2'. After verification that 'R1' was a clear solution (storage at/or near to 4° C may lead to crystallisation; this could be rectified by slight mixing at room temperature of the reagent, prior to use) 6 ml of 100 % methanol was added to the 18 ml bottle of reagent 'R1'. Before each series of measurements, the baseline of the spectrophotometer was adjusted (absorbance zero) with water-filled cuvettes. All solutions were stored between 4 and 10 °C until needed. The assay procedure was as follows:

1. 600 µl of freshly prepared 'R1' final solution was pipetted into each of the reaction test tubes. Reagent 'R1' contains a chromogenic reagent at the concentration of 11.4 mM, in acetonitrile.
2. For each assay, 200 µl of aqueous seed extract was then added and mixed thoroughly with 'R1'.

3. The reaction was started by adding 150 µl of 10.4 M methanesulfonic acid reagent 'R2'. This was then thoroughly mixed and the test tube closed with a tight screw-top stopper.
4. The reaction mixture was then incubated for 40 mins in a 45 °C water bath.
5. Finally the absorbance (A) was read at 586 nm. For each series of assays, a control sample in duplicate was included ([aldehyde]=0) whose mean absorbance value (A°) was further subtracted from the (A) values obtained in the presence of the tissue samples.

2.5.1 *Standards*

The first standard 'solution S1' was a solution of 10 mM 4-HNE as the diethylacetal, in acetonitrile (1 ml). The second standard 'solution S2' was a solution of 10 mM of 1,1,3,3-tetramethoxypropane in 20 mM Tris-HCl buffer, pH 7.4 (1 ml). Standard solutions 'S1' and 'S2' were available for the preparation of standard curves of 4-HNE and MDA, respectively. Before use, these solutions were diluted 100-fold (v / v) in the same medium as that used for the sample dilution (i.e. phosphate extraction buffer). This resulted in 100 µM concentrations of either aldehyde standard. Standard solutions were always freshly prepared. The sample was replaced by 0 to 200 µl of 100 µM standard solution, adjusted with buffer to a final volume of 200 µl, i.e. with the standard concentration range of 0 to 20 µM in the final reaction medium.

2.5.2 *Calculation of concentration*

The following equation was used to calculate the concentration (M) of MDA and 4-HNE in the samples:

$$[\text{MDA} + \text{4-HNE}] = (A - A°) \times 5 / \text{extinction coefficient}$$

2.5.3 *Sensitivity and reproducibility*

The sensitivity of the assay was high, but the detection threshold appeared to be where 0.5 μM of aldehyde or lower was present in the final reaction medium. In practice, 0.5 μM was considered as the lower limit for the assay. For sample volumes of 200 μl, the lower limit of measurable MDA or 4-HNE within the sample was therefore approximately 2.5 μM. Reproducibility was determined by conducting repeat assays over a 5 day period, using the same experimental conditions. Running standard stock solutions of 'S1' and 'S2' stored at 4° C, the standard error values were lower than 5 % at $P > 0.05$.

2.5.4 *Adaptation: specific assay of MDA*

The assay of MDA could be performed by replacing the methansulfonic acid (reagent 'R2') with the same volume of 37 % aqueous HCl during the above procedure. The reaction then required 60 minutes to reach a plateau when monitored at the 586 nm wavelength. The assay was then specific to MDA due to the very weak reactivity of 4-HNE in acidic conditions.

2.5.5 *Calculations*

A combination of the two procedures (4-HNE and MDA), including two samplings and two distinct assays, was then used to derive the respective concentrations of MDA and total 4-HNE. The apparent molar extinction coefficient obtained with MDA was the same for both procedures.

2.6 Determination of lipid peroxides using the K-assay™ (LPO-CC) Kit

This commercial assay kit (Kamiya Biochemical Company, Tukwila, U.S.A.) is for the quantitative determination of lipid peroxides in serum, plasma and other biological materials. This assay is reported to have two major advantages over other assays for lipid peroxide concentrations:

- It specifically quantitates lipid peroxides, and does not measure the metabolites of lipid peroxides.
- The procedure can be either manual or automated.

Lipid peroxides are generally produced by the autoxidation of unsaturated fatty acids, in biological specimens. The quantitative determination of lipid peroxides is believed to be useful for checking the disease state and prognosis of various diseases in humans (such as liver diseases and diabetes). As lipid peroxides can be very unstable in biological material, and be present in very low quantities. It is necessary (particularly in the medically related fields), to directly determine lipid peroxide levels by a quick and simple technique. The K-Assay™ LPO-CC is one such technique.

2.6.1 *Principle*

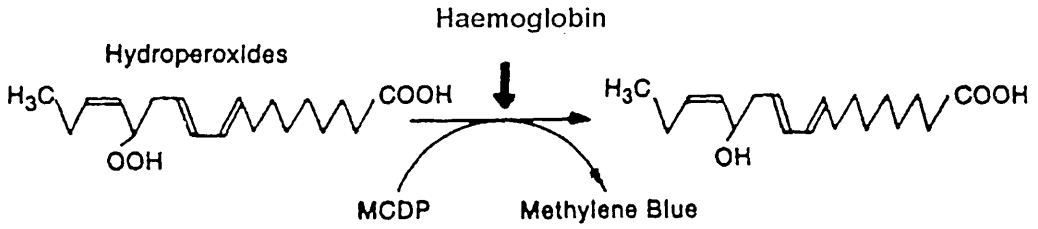
In the presence of haemoglobin, lipid hydroperoxides are reduced to hydroxyl derivatives (lipid alcohols) and the 10-N-Methylcarbamoyl-3,7-dimethylamino-10 H-phenothiazine (MCDP) chromogen reagent present in the assay is oxidatively cleaved to form methylene blue in an equi-molar reaction. Lipid peroxides can then be quantitated by colourimetrically measuring the methylene blue formation at 675 nm (see diagram on page 65).

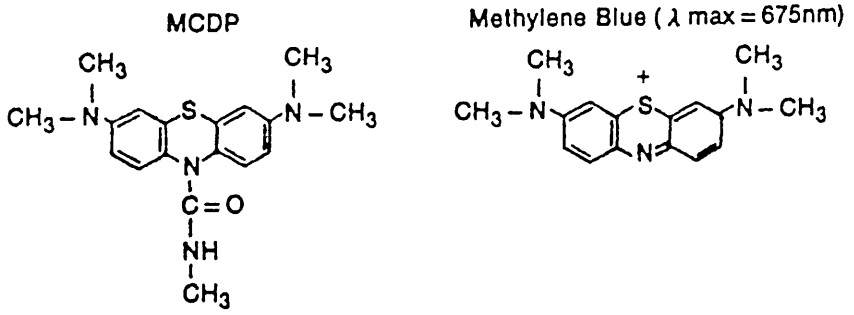
2.6.2 *Reagent Preparation*

To prepare reagent 1 (R1): Reagent 1A (lyophilised ascorbate oxidase / lipoprotein lipase) was dissolved in the provided buffer solution 1B (Good's buffer), and incubated for 15 min, at 37 °C after dissolving. Reagent 2 (R2) was prepared by dissolving reagent 2A (lyophilised MCDP) in buffer 2B (Good's buffer containing heamoglobin), and incubated for 15 min, at 37 °C after dissolving. The unconstituted and reconstituted reagents were stored at 2 - 8 °C, and protected from light by wrapping the vials in aluminium foil. The shelf life of reconstituted reagents was extended by aliquoting and freezing the reagents at -20 °C.

2.6.3 *Stability*

Unopened reagents are reported to have a shelf life of 1 year from the date of production if stored at 2 - 8 °C (Kamiya Biochemical Co., 1996). Dissolved (reconstituted) reagents were stable for 3 days when stored in the dark at 2 - 8 °C. If aliquoted, they could be stored in the dark at -20 °C for up to 2 - 3 months.





MCDP: 10-N-Methylcarbamoyl-3, 7-dimethylamino-10 H-phenothiazine

2.6.4 *Interference*

EDTA is reported to affect the assay, but the small amount used in the extraction buffer did not cause interference. Exposure of the reagents to light, dust, $NaClO_4$, and metal ions such as Cu, Fe, and Co are also reported to interfere with the assay, and were avoided.

2.6.5 *Procedure*

The assay procedure was as follows:

1. 1.0 ml of 'R-1' was mixed with 0.1 ml of the sample (S).
2. To prepare a calibration curve, 1.0 ml of 'R-1' was also mixed with 0.1 ml of cumene hydroperoxide standard (Std) and 0.1 ml of saline blank.

3. The reaction mixture was then incubated at 30 °C for at least 5 minutes.
4. 2.0 ml of 'R-2' was added to each test tube, and mixed.
5. The reaction mixtures were then incubated at 30 °C for at least 10 min, by which time the reaction was complete.
6. Finally, optical absorbance was measured at 675 nm of sample (Es), Standard (Estd) and blank (Eb). The sample and reagent volumes could be changed provided; the sample : reagent ratio remained 1:30 and the R1 : R2 ratio remained 1 : 2.

2.6.6 *Calibration curve*

A simple two point line of calibration was constructed using a saline blank (0 mg / l) and the provided 50 nmol / ml cumene hydroperoxide standard, as recommended by the manufacturer. It seems that the standard solution provided cannot be diluted, and hence only a two point line can be accurately constructed.

2.6.7 *Quality control*

Controls were run with each batch of samples to monitor the procedure. The values obtained for the controls fell within the manufacturer's specified range.

2.6.8 *Calculations*

Calculation of the total amount of lipid peroxide was made through the equation:

$$[\text{Total lipid peroxides}] = (Es - Eb) / (Est - Eb) \times 50.0$$

2.6.9 *Limitations*

The measuring range for this assay was found to be between 2.0 nmol / ml and 300 nmol / ml of lipid peroxides for accurate quantitative determination.

2.7 Antioxidant determination

The measurement of antioxidant status is an indirect means of predicting how efficiently the seeds metabolism will be able to control excessive free radical / lipid peroxidation activity. As a number of environmental factors can influence levels of antioxidants in tissues, these studies were carried out in conjunction with assays which measured levels of actual breakdown products of free radical activity (see last section).

The majority of the assay procedures, including those used in this investigation for antioxidant status involve substrate / enzyme reactions detectable by UV / visible spectroscopy.

2.7.1 Preparation of bovine serum albumin standards

Protein standards were made by diluting a stock bovine serum albumin (BSA) reagent in the same phosphate buffer diluent as the sample whose protein concentration was to be determined.

2.7.2 Determination of soluble protein concentrations using the Coomassie assay

2.7.2.1 *Introduction.*

The Coomassie protein assay is a ready-to-use Coomassie blue G-520 reagent solution for the quantitative determination of total protein concentration. The assay reagent is based on the Bradford method which utilises an absorbance shift in acidic Coomassie brilliant blue G-250 solution (Diamant *et al.*, 1967).

Upon addition to a solution containing protein, the dye binds to the protein resulting in a colour change from a reddish brown to blue. The dye has been assumed to bind to protein via electrostatic attraction of the dye's sulfonic groups (Compton and Jones, 1985).

Another hypothesis suggests the Coomassie Blue Reagent exists in three equilibrium states, anionic, neutral, and cationic with peak spectrophotometric absorbances at 595, 650, and 470 nm respectively. The anionic species is believed to be bound to the protein based on an absorbance shift from 465 to 595 nm that occurs when Coomassie blue reagent binds maximally to arginine and weakly to histidine, lysine, tyrosine, tryptophan and phenylalanine residues. It has been suggested that Coomassie blue binds proteins that have basic and aromatic functions and macromolecular structure (Compton and Jones, 1985).

2.7.2.2 *Standard assay procedure*

Preparation of a known concentration of protein was conducted using the diluted stock BSA standard. Convenient standard concentration points were; 75, 100, 250, 400, 500, and 1000 µl / ml. The assay was then conducted as follows:

1. 0.1ml of diluted BSA standard or aqueous seed extract was pipetted into a 16 x 100 mm reaction tube (using the phosphate buffer as the 'blank').
2. 5.0 ml of Coomassie protein assay reagent was then added and mixed well.
3. Absorbance was read at 595nm within 90 min against deionised water.

Absorbance at 595 nm of the 'blank' was subtracted from each standard or unknown protein sample absorbance, and a standard curve was prepared by plotting the average net absorbance at 595 nm for each diluted BSA standard. Using the standard curve, the protein concentration for each unknown protein sample was determined.

The dye solution consists of Coomassie brilliant blue G-250, phosphoric acid, methanol, water and solubilising agents. Small quantities (1 ml) of albumin standard were used, containing fraction V bovine serum albumin in 0.9 % NaCl with sodium azide (the protein concentration was 2mg / ml).

2.7.3 Determination of peroxidases

2.7.3.1 *Solutions required*

The following solutions were needed to conduct the assay; 0.05 M phosphate buffer adjusted to a pH of 6.1, added to this just before use was 16 mM of guiacol and 2 mM hydrogen peroxide.

2.7.3.2 *Initial preparation of guiacol*

Guiacol is a colourless liquid at room temperature, but has a very strong aromatic odour, and hence use of the substance was strictly restricted to the fume cupboard. A 16 mM solution of guiacol was prepared in 100 ml of phosphate buffer.

2.7.3.3 *Preparation of hydrogen peroxide*

The stock solution of hydrogen peroxide was 30 % (v / v), for a 100 % (1M) solution of hydrogen peroxide 34 g was dissolved in a litre of distilled water. The assay required a 2 mM solution, which equated to 20.4 µl H_2O_2 being added to 100 ml of phosphate buffer solution.

2.7.3.4 *Assay procedure*

The following procedure was carried out on ice quickly, to minimise the effects of degradative processes that may occur in the aqueous seed extract. 20 µl of aqueous seed extract was then added to 980 µl of the reaction mixture. This was mixed thoroughly in the cuvette and the orange pigment forming from the oxidised guiacol was followed spectroscopically at 470 nm.

The reaction measurement was conducted at 25 °C, a chart speed of 30 mm / min with an absorbance maxima of 0.5 OD units. The time of measurement was typically 2 mins. The results were expressed as a rate of change of absorbance at 470 nm / min / mg protein (corrected to 1 ml).

Example of 3 reps.

Chart speed = 0.5 mm / sec

1 mm = 2 sec 100 µl / 20 µl = 50

average = 0.035 absorbance units

Abs max = 1.1

Abs min = 1.0 if the absorbance changes by 0.035 units in 40 seconds

The total absorbance change = 0.1 units

= 0.035 / 40 x 60 = 0.653 / min

0.653 x 50 = 32.65 min^{-1} / mg / ml

2.7.4 Determination of catalase

2.7.4.1 *Procedure*

The following procedure was carried out quickly on ice, again to minimise the effects of degradative processes that may occur in the aqueous seed extract. A spectrophotometer was used at 240 nm (UV region of the spectrum) with white filtering. 2.9 ml of substrate (diluted H_2O_2; 1 ml of 30 % solution in 400 ml

phosphate buffer) was added to quartz cuvette. 0.1 ml of aqueous seed extract was then added to the same cuvette, and the change in absorbance recorded over time.

2.7.4.2 *Calculations*

Calculation of the results was as abs / min / ml (see sample calc), or (abs / min x 1000 x dilution factor x 3) / (43.6 x 2). The dilution factor was calculated in the following way;

2 ml enzyme = 1, therefore 1 ml = 2 / 1 = 2

0.5 ml = 2 / 0.5 = 4

0.1 ml = 20

0.01 ml = 200

0.02 ml = 100 in chamber

Sample calculation; for a change in absorbance at 240 nm / min / ml.

If the chart speed = 30 mm / min

= 1 mm / 2 sec

Abs max = 1.1

Abs min = 1.0

If the change in absorbance = 0.1 units, over a period of 20 mm

20 mm x 2 = 40 secs

0.035 abs in 40 secs

0.035 / 40 x 60 = 0.0653 abs / min

a dilution factor of 50 µl in 1000 µl, is equal to multiplying the answer by 20.

0.0653 x 20 = 1.06 abs / min / ml.

2.7.5 Determination of superoxide dismutase using the SOD-525 assay kit

The SOD-525 method provided a simple, reproducible and fast tool for the assay of SOD activity, and required only one sampling per assay.

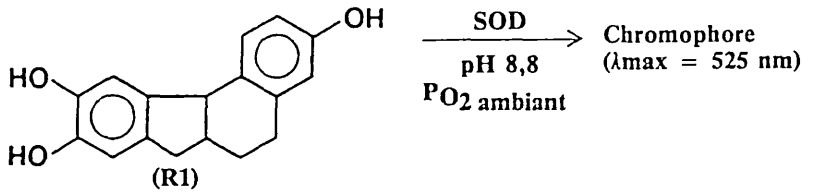
2.7.5.1 *Principle*

Superoxide dismutases are mettaloenzymes which catalyse the dismutation of the superoxide ion into oxygen and hydrogen peroxide, according to the following reaction:

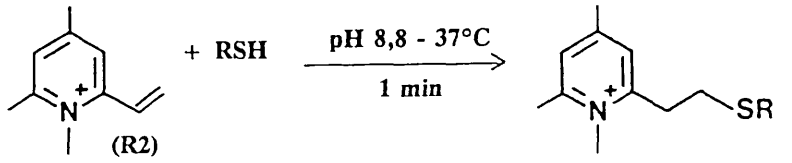
$$2\ O_2^{\cdot-} + 2\ H+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

It has been widely suggested (see Chapter 1) that such enzymes provide a defence system which is essential for the survival of aerobic organisms (Beyer *et al.*, 1991). Numerous methods have been developed during the last few years to assay SOD activity, but most of the reproducible and more sensitive are difficult to standardise.

The SOD-525 method takes advantage of a proprietary reagent 'R1', whose alkaline autoxidation is accelerated by any catalyst endowed with SOD activity. This autoxidation yields a chromophore which absorbs visible light at 525 nm. The reagent 'R1' has been patented (Patent No.92 02082) by Bioxytech, S.A.



This chromophore is only stable for a few minutes. The SOD-525 method also takes advantage of a second proprietary reagent 'R2' (Patent No. 91 14782) which allows the elimination of major interferences due to mercaptans in the sample, such as glutathione (represented by RSH in the following diagram). Reagent 'R2' traps such mercaptans by means of a rapid alkylation reaction, thus:



The pH at which the kinetic measurement is performed was fixed at 8.8, which results in optimal sensitivity of the assay without significant inactivation of known SOD enzymes such as, for instance, copper/zinc-, manganese- or iron-SOD. The assay is known only to be affected by certain chemicals which are listed in Table 2.3.

Each kit contains the following chemicals:

- 'R1' (2 x 3.3 ml bottle) solution of cromogenic reagent R1 in 3.2 x 10 - 2 M HCl.
- 'R2' (2 x 3.3 ml bottle) solution of R2 (mercaptan scavenger) in Dimethylsulphoxide (DMSO) containing 25 % (w / v) ethylene glycol.
- 3 (2 x 100 ml bottle) Buffer solution titrated at pH 8.8 (at 37 °C), which contains 0.11 mM diethylenetriaminepentaacetic acid (DTPA)

The three solutions were ready for use, straight from the packet. All solutions were stored between 2 and 8 °C, and were stable at this temperature for approximately 6 weeks.

2.7.5.2 *Procedure*

2.7.5.2.1 *Preparation of samples*

The SOD-525 method can be used with any aqueous solution, whether its origin is biological or not, provided that the sample concentration in the reaction medium results in a measured rate of autoxidation of 'R1', i.e. a Vs, which:

1. Is not perturbed by interference.
2. Does not yield a Vs / Vc ratio lower than 1, where Vc stands for the rate of autoxidation 1 of R1, in the absence of the sample (control).

It was checked, therefore that the SOD activity in the sample was not underestimated by the presence of interfering reagents in the reaction mixture. For this purpose, two

Table 2.3
Interfering agents known to affect the Bioxytech, S.A. SOD-525 assay, together with the concentration threshold below which no prior sample preparation is required, and if necessary the appropriate method of elimination.

Interfering agent	Concentration threshold (µM)	Elimination procedure
Heamoglobin	**0.5**	**P1**
Albumin	**0.1**	**P1**
Ascorbic acid	**0.5**	**P2**
NAD(P)H	**10**	**P2**
BHT	**30**	**P2**

- **P1**: 40 µl of ice-cooled absolute ethanol/chloroform 62.5 / 37.5 (w / v) can be added to 250 µl of sample in a glass tube. This is then thoroughly mixed for at least 30 seconds and centrifuged at 4 °C and 3,000 rpm for 5 minutes. The resulting supernatant can then be taken and transferred into a second tube and stored between 2 and 8 °C, and can be used for the assay.

- **P2**: The sample needs to be diluted with water and checked that 2 different dilutions give the same final result. If this is not the case, the process needs repeating until the discrepancies have been resolved. If this is not possible, ascorbic acid must be separated from the catalyst to be assayed by means, for instance, of a user-ready gel-filtration cartridge with an appropriate molecular weight cut-off.

Table 2.4
Outline of the initial preparation of the Bioxytech, S.A. SOD-525 assay reactants, prior to measurement, (*) the distilled water underwent the same process as that of the sample.

	Control	Sample
Solution of 'R2'	**30 µl**	**30 µl**
Distilled water (*)	**40 µ**	-
Sample	-	**40 µl**
Solution 3	**900 µl**	**900 µl**

assays were performed on two distinct sample dilutions, to verify that the results were the same when the dilution factors were taken into account.

2.7.5.2.2 *Assay procedure*

The measurement parameters were: a final pH (at 37 °C) of 8.8, buffer equilibrated at 37 °C at the ambient oxygen concentration. (Only the amount necessary for the assay to be performed was equilibrated). For each measurement, the reaction medium was prepared immediately prior to measurement as described in Table 2.4.

The reaction mixture was then thoroughly mixed for 1 minute at 37 °C. 30 µl of solution 'R1' was added, mixed, and immediately the absorbance at 525 nm recorded against air (reference cuvette). Absorbance was monitored for a 1 minute period. There was no more than a 10 second duration between the addition of 'R1' and the start of monitoring.

For each measurement, the reaction rate was determined from a calculation of the maximal slope of the absorbance time-course (over about 30 s). The slope reflects a rate of 'R1' autoxidation which was linear. This was visually checked. Results were expressed in absorbance units per minute.

2.7.5.3 *Calculations of SOD activity*

Vc and Vs were the experimentally determined reaction rates of the control and the sample, respectively. The SOD activity of each sample was directly obtained from

the experimental Vs / Vc ratio, through the inspection of a SOD-525 conversion table supplied with the kit (see appendix IV).

The resulting value was multiplied by the dilution factor of each sample. Hence, the results are expressed in SOD-525 activity units per ml of sample. Values in the SOD-525 conversion table have been deduced from the following rate equation:

$$Vs / Vc = 1 + [SOD] / a [SOD] + b, \text{ with } a = 0.073 \text{ and } b = 0.93$$

The SOD-525 unit defined by Bioxytech S.A. corresponds to a Vs / Vc ratio which is equal to 2 in the above conditions. A standard curve was also constructed during the investigation (for the purpose of checking) from serial dilutions of an initial SOD standard of unknown activity. Ideally, such serial dilutions should result in Vs / Vc ratios included within the 2 to 8 range. It was an absolutely necessity to evaluate the control rate (Vc) as the mean value of four independent measurements. Whichever type of measured SOD, a universal standard curve is obtained (Fig 2.1).

2.7.5.4 *Accuracy, reproducibility and sensitivity*

When a series of 30 measurements were performed on the same day using the same experimental conditions, in the absence (control) or presence of SOD at 3 concentrations covering the full range of the standard curve, the standard errors on (Vs / Vc) mean values are all lower than 5 % ($P > 0.05$). When the previous experiment is performed three days later, the new standard error values calculated on the two measurement series are again all lower than 5 % ($P > 0.05$).

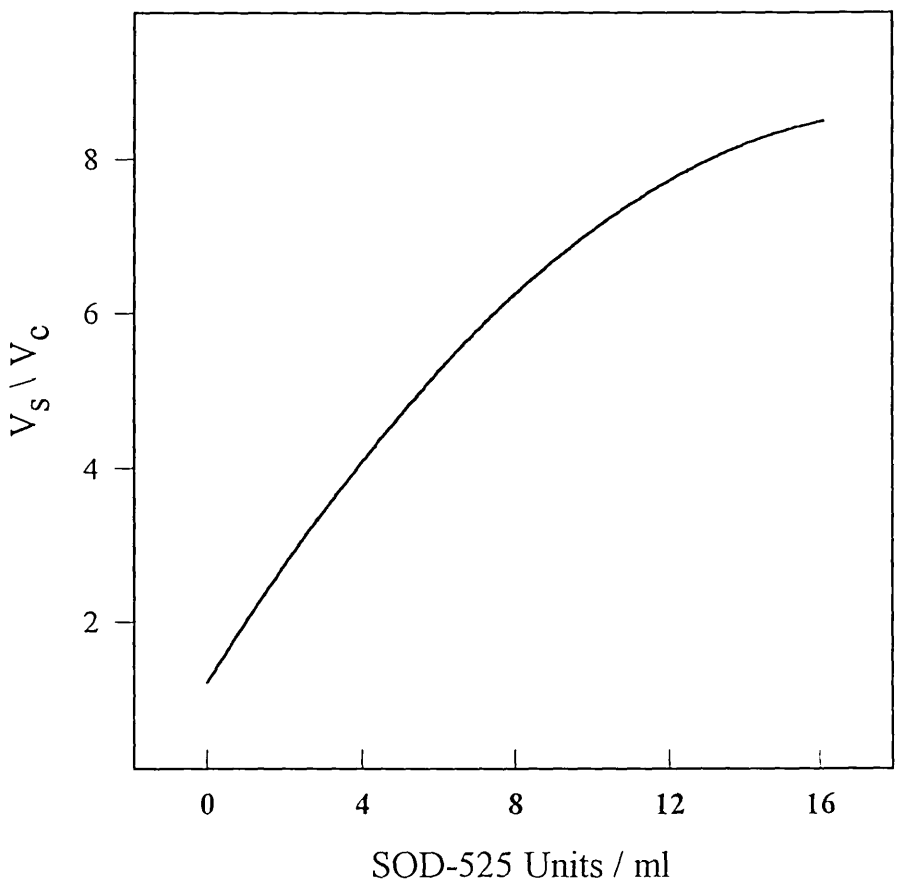


Figure 2.1 Universal standard curve obtained using the Bioxtech, S.A. SOD-525 assay kit. Serial dilutions resulted in Vs / Vc ratios within the recommended 2 to 8 range. Control rate (Vc) is the mean value of four independent measurements.

From 30 repeated measurements performed on the control within a single day, all in the same experimental conditions, the detection limit of the assay was found to be 0.2 units per ml.

2.8 Measurement of seed respiration and membrane integrity

2.8.1 Measurement of seed respiration

Oxygen uptake of seed tissue components was determined using a Gilson submarine single valve differential respirometer (Gilson Medical Instruments, Wisconsin, U.S.A.; Gilson, 1963 and Umbreit *et al.*, 1972). This was determined independently of carbon dioxide evolution by employing an alkali trap of 10 % KOH to absorb the latter. The system was allowed to openly-vent for 10 min after each experimental run to allow for changes in gas pressure.

2.8.2 Assessment of membrane integrity - electrical conductivity

Samples of seed tissue were prehydrated for 15 min on damp filter paper to alleviate imbibitional and / or chilling damage, then soaked individually in 12 ml of distilled water. Conductivity of the steep water was determined every 30 min for 3 h using a MC1 Mk V portable electrical conductivity meter (Electronic Instruments, Chertsey, U.K.). Five replicates of each tissue were used per treatment.

2.9 Determination of total unfrozen water fractions

2.9.1 Design and principles of technique

Whenever a material undergoes a change in physical state, such as a crystallisation or melting of water, heat is either absorbed or liberated. Many such processes can be initiated by raising or lowering the temperature of the material. Differential Scanning Calorimeters (DSC) are designed to determine the enthalpies of these processes by measuring the differential heat flow required to maintain a sample of the material and an inert reference at the same temperature.

In a typical DCS system the sample and reference chambers are each provided with individual heaters making it possible to use a 'null-balance' principle (McNaughton and Mortimer, 1975). The system is divided into two control loops, the first for average temperature control so that the temperature of the sample and the reference may be increased or decreased at a pre-set rate. The second loop ensures that if a temperature difference develops between the sample and reference, because of an exothermic and endothermic reaction on the sample, the power input is adjusted to remove this difference, resulting in a 'null-balance'. Therefore, the temperature of the sample holder is always kept the same as that of the reference holder by continuous adjustment of the heater power. A signal proportional to the difference between the heat input to the sample and that to the reference, is fed into the DSC computer, which produces a graphical representation, and also registers other experimental conditions such as average sample and reference temperature.

2.9.2 Differential scanning calorimetry

A power compensated Perkin Elmer DSC-7 differential scanning calorimeter (Perkin Elmer Thermal Analysis, Berkshire, U.K.) was used to measure un-frozen water contents within seed material. The CCA7 temperature controller was set at -120 °C throughout operation, with nitrogen gas at 30 psi to operate the valve controlling the flow of liquid nitrogen, cooling the measurement head. Helium was used at 20 psi as the purge gas.

2.9.2.1 *Method of determination and calibration protocol*

Samples (10 - 30 mg f. wt) were weighed into pre-weighed 10 µl aluminium pans and a lid immediately crimped on top. These were then loaded into the sample chamber at 25 °C, an empty aluminium pan was used as the reference chamber. The DSC-7 was calibrated for each run using an empty sample pan at 20 °C min^{-1} with indium and octane, and checked with cyclohexane (tabulated melting points 156.6, -56.76 and 6.54 °C respectively). Enthalpy calibration was performed using indium (28.45 J g^{-1}).

Samples runs followed the protocol described below at 20 °C min^{-1};

The first cool ran from 25 → - 100 °C, the first heat from - 100 → 25 °C. The runs were then repeated and the instrument finally returned to 25 °C.

The traces produced were analysed using software supplied with the DSC-7. The onset, endpoint and height of 1st order transitions (peaks) were measured with the

baseline being extrapolated from that following the melt (Richardson, 1993), from which the total un-freezable water fractions were calculated.

2.10 Statistical analysis

The statistical package GLIM version 4.0 (Healy, 1988) was used for the comparison of multiple regression lines. Analysis for statistical significance of the increase in scaled deviance caused by contrasting multiple regression lines to have the same slope and / or same origin, used the *F*-distribution (Crawley, 1993). Kruskal-Wallis one-way ANOVA was used to compare multi-group non-parametric data with unequal sample sizes (Zar, 1984), using the computer package Unistat (version 4.0) for Microsoft Windows (version 3.x and 4.0 / 95) for Intel Pentium based personal computers. A simple chi-squared test was used to compare multi-group non-parametric data of equal sample size.

CHAPTER 3

Determination of Oxidative Stress in Seeds in Relation to Desiccation Injury - An EPR Study

Chapter 3: Determination of oxidative stress in seeds in relation to desiccation injury - an EPR study

Outlay of Chapter

Chapter 3: Determination of Oxidative Stress in Seeds in Relation to Desiccation Injury - An EPR Study

3.1 Introduction

Roberts and King (1980) first emphasised that the classification of seeds as recalcitrant needed further investigation. This view emanated from the finding that *Citrus* spp. which were originally described as such, were in fact able to survive desiccation and storage at sub-zero temperatures (storage greatly extending the time required for seeds to germinate in the viability test; as reviewed by Hong and Ellis, 1995).

Although many seed systems have been studied (using biophysical, biochemical methods etc., see section 1.2.1), to date little is known about the cause of sensitivity to desiccation in recalcitrant seeds, or the mechanisms of viability loss. One possibility, proposed by Hendry *et al.*, (1992), is that during moisture stress recalcitrant seeds become exposed to activated forms of oxygen and subsequently accumulate 'stable' free radicals, and products of lipid peroxidation, during what has been termed a period of 'oxidative stress'.

The role of oxidative chemistry in the stress responses that seeds exhibit during storage, ageing (both natural and artificial) and desiccation remains illusive. Although there have been many studies which have demonstrated an oxidative

response upon desiccation (see section 1.2.1.3), the results are generally inconclusive and those between different research groups have on occasion been contradictory (e.g. Buchvarov and Gantcheff, 1984 and Priestly *et al.*, 1985).

Variance in results may have stemmed from the use of different experimental procedures applied to a varied range of material exposed to very different treatments. Oxidative activity is no doubt dependent upon the effects of such treatments, along with other factors including; the initial hydration status of the seed, the part of the seed under investigation, the batch of seed investigated, its species and variety.

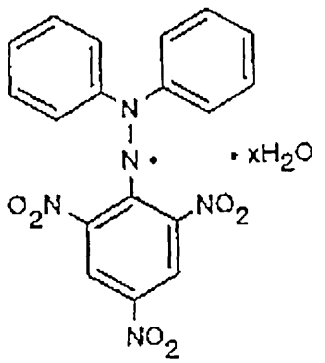
Therefore, the techniques used to investigate damaging oxidative events in seeds, require careful consideration and assessment, and only by using a range of tests will one begin to obtain a meaningful estimate of oxidative activity (Benson, 1990). All such tests require close scrutiny and evaluation before being routinely used.

Oxidative injury can be determined in biological systems by either direct or indirect measurement. Indirect measurement quantifies the intermediates and products of lipid peroxidation. Direct measurement, however, can only be achieved by electron paramagnetic resonance (EPR) determination of free radicals (formerly known as electron spin resonance - ESR). This chapter describes parameters which were found to affect the applicability of the EPR technique, to the investigation and evaluation of oxidative stress in desiccating recalcitrant seeds.

3.2 Experimental design

3.2.1 EPR detection of free radicals: influence of water

The validity of using EPR as a technique for determining free radical activity within seed material of differing moisture contents was investigated using a chemical which constituted a 'stable' free radical. DPPH (2,2-diphenyl-1-picrylhydrazyl) was chosen for this purpose because it is relatively stable over a range of experimental conditions and temperatures, and has been commonly used in EPR investigations as a g-value marker (Knowles *et al.*, 1976). DPPH is a deep violet aromatic, with a melting point of 130 - 133 °C, its molecular structure is shown below.



To ensure that the experimental conditions were as close as possible to those to be used in seed desiccation investigations, a suitable matrix (substrate) within which the DPPH crystal could be mixed had to be sought. Cellulose powder was chosen as it was an inert plant derived substrate. One crystal of DPPH was mixed with 40 μg of cellulose powder. This was then placed carefully in a Suprasil quartz tube (c. 3 mm diameter), and 'tapped' to the bottom of the tube. The tube was then stoppered and

placed reproducibly in the spectrometer cavity. Measurements were conducted at both ambient (20 °C / 293 K) and liquid nitrogen (-196 °C / 77 K) temperatures.

After these initial measurements 5µl aliquots of double-deionised water were sequentially added by syringe to the dewer at room temperature, using a hypodermic needle to 'drop' the water onto the top of the powder mixture. The tube was then again gently 'tapped' to ensure that the water was fully absorbed into the powder mixture, and left to equilibrate for 10 mins. Measurements were then re-conducted, at room temperature and then at liquid nitrogen temperatures. Other measurements were made in the same way after the further addition of aliquots of water, up to a final amount of 40 µl. Target moisture contents (on a f. wt. basis) for the samples were 0, 10, 20, 27, 33, 38, 43, 47 and 50 %. Gravimetric moisture content analyses were made of duplicate samples, and were found to agree after a comparison was made using a chi-squared test ($r = 0.85$; $P < 0.05$).

3.2.2. Detection of free radicals in seed material

The ability to detect free radicals using EPR in a range of seed material was of paramount importance to this study. A range of temperate recalcitrant seed material was selected and determinations made at the tissue component level. Seeds of *Aesculus hippocastanum* L. (horse chestnut), *Quercus robur* L. (pendunculate oak) and *Quercus rubra* L. (red oak) seeds were desiccated for up to 80 h in the dry room, with samples being removed at regular intervals along their full drying curve. The seeds were separated randomly into three groups; 30 for germination studies ($n = 2$ x 15); 7 for moisture content determination; and the remaining 5 were dissected, the

embryonic axes removed, and rapidly-frozen and stored immediately in liquid nitrogen for subsequent EPR analysis.

3.2.3 Effects of tissue quality and pigmentation on EPR signal intensity

It was noted that during routine cold storage of pendunculate oak seeds, that some embryonic axes had developed necrotic areas as the quality of the seeds was lost. To establish how this type of hydrated senescence might affect the EPR determinations, oak axes at three levels of necrosis (0, 50 and 100 %) were investigated. All classes of necrosis (as two replicate lots) were studied at both ambient and liquid nitrogen temperatures.

To investigate whether seed pigmentation might interfere with the interpretation of EPR data, the tropical recalcitrant species *Azadirachta indica* A. Juss (neem) and *Carica papaya* L. (papaya) were examined. Neem seed has a dark brown pigmented mesotesta which surrounds the embryo, and papaya has a heavily pigmented testa, as such both species provided a good model system. First derivative scans were taken sequentially in neem of the whole stone (endocarp in place); of the seed including the pigmented mesotesta, and of the 'naked' embryo (the mesotesta was carefully removed under liquid nitrogen using a scalpel, as at this temperature it tended to neatly flake off). Papaya seeds were investigated using both first and second derivative scans of the seed testa, and the 'naked' seed with testa removed.

3.3 Results

3.3.1 EPR detection of free radicals : influence of water

After tuning of the instrument to ensure that the EPR cavity was correctly coupled (i.e. all of the microwave power incident on the cavity was being absorbed), scans of DPPH were produced with a high signal : noise ratio. The spectra obtained at 20 °C show several interesting features (Fig 3.1). Firstly, the signal obtained from the DPPH was strong (in the absence of water) and constituted a single sharp peak with a g-value of 2.0037 ± 0.0005. Secondly, it was clear that when water was added to the system, the signal intensity reduced dramatically, the signal being almost completely lost when 30 µl of water was added (equivalent to 38 % moisture content; f. wt. basis). Indeed, adding water reduced the deflection of the signal from a relative mm value in Fig 3.1 of 95 to between 1 and 2.

When the experiment was conducted at -196 °C (Fig 3.2), the signal (g-value remaining constant) characteristics did not systematically change greatly upon desiccation. In both dry and 'wet' conditions the single peak signal intensity remained high, varying from 52 to 69 mm (relative units of deflection). At room temperature, an approximate 18-fold increase in signal intensity was observed, as moisture content was decreased from 38 to 0 % (Fig 3.1), whereas at -196 °C, the mm deflection over this range was relatively constant at 62 ± 7 (s.d) mm (Fig 3.2). Moreover, the latter measurements benefited from an increase in the signal : noise ratio. There was a slight reduction in signal intensity as water was added (between 5

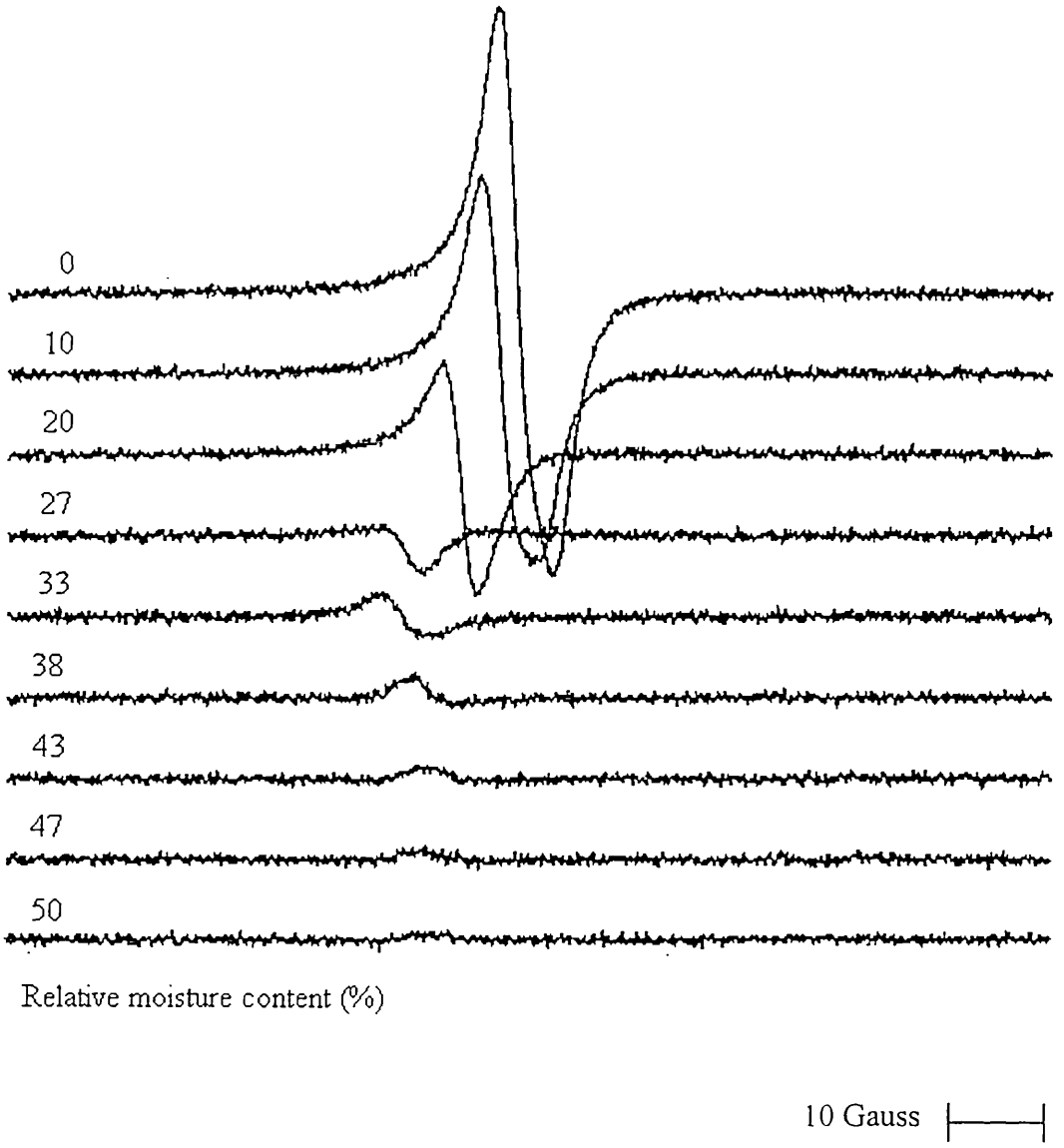


Figure 3.1

First derivative EPR spectra showing the single peak signals derived from a single crystal of 2,2-diphenyl-1-picryhydrazyl (DPPH) in 40 μg of cellulose powder at ambient (20 °C) temperature, using the surviving-tissue technique (receiver gain = 2.5e + 05). The nine scans shown were performed at a range of increasing moisture contents, running from 0 to 50 % (f. wt basis).

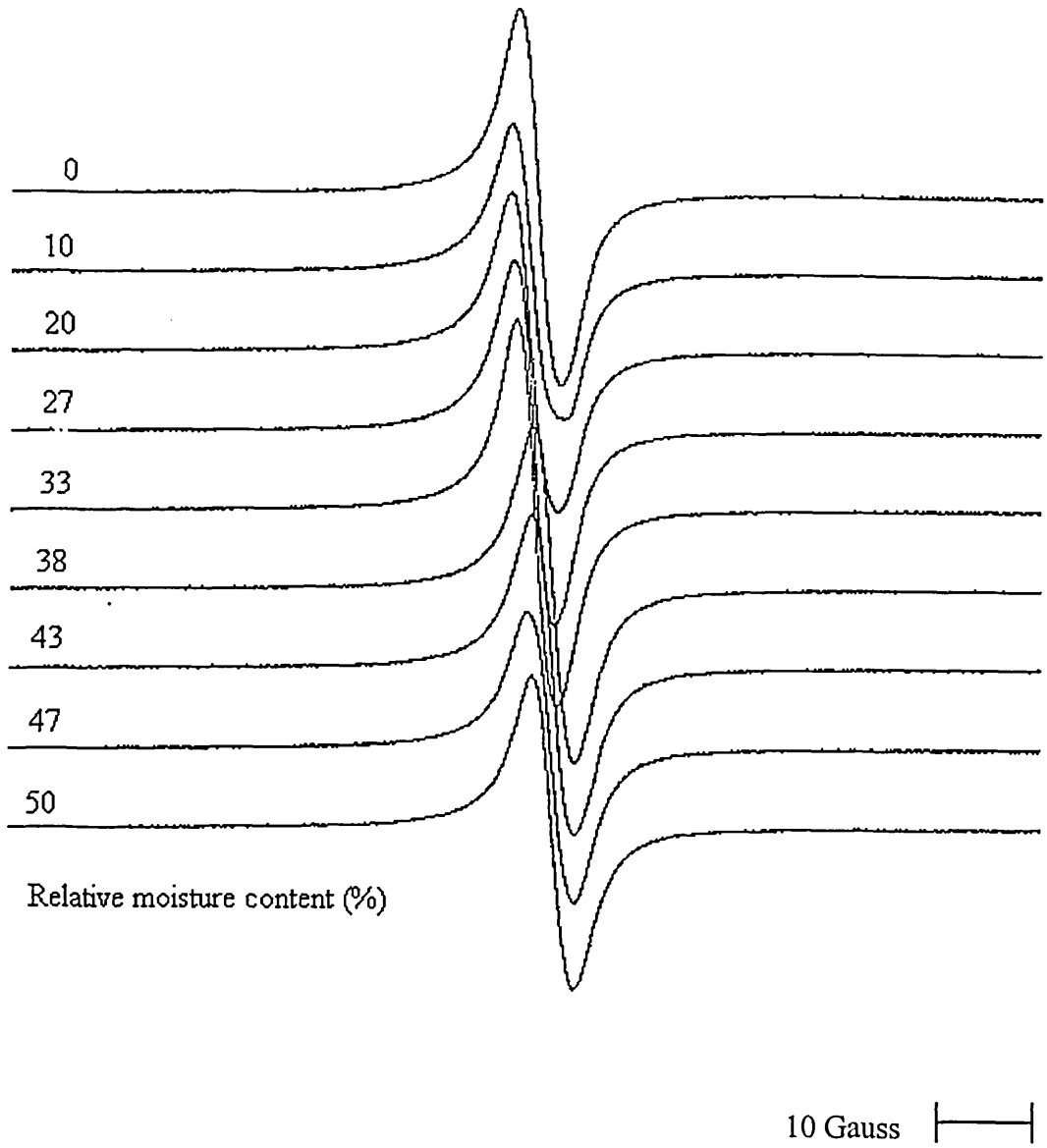


Figure 3.2

First derivative EPR spectra showing the single peak signals derived from a single crystal of 2,2-diphenyl-1-picryhyrazyl (DPPH) in 40 μg of cellulose powder using the rapid-freeze (-196 °C) technique (receiver gain = 2.5e + 05). The nine scans shown were performed at a range of increasing moisture contents running from 0 to 50 % (f. wt basis).

and 7 mm) but a chi-squared test demonstrated that this was not significant ($r = 0.85$; $P < 0.05$).

3.3.2 Detection of free radicals in seed material

First derivative scans using fresh axes of red oak (Fig 3.3A), horse chestnut (Fig 3.4A) and pendunculate oak (Fig 3.5A) at -196 °C revealed a strong free radical signal was present in all, although the signal was smaller than that observed for DPPH. Second derivative scans revealed more information about the free radical signal, indicating both a low field (LF) and a high field (HF) component (Figs 3.3B, 3.4B and 3.5B). Also the relatively lower signal intensities typical of seed material were easier to observe; note the receiver gain for second derivative scans is about half of that of the first derivative scans, but the deflection is similar.

Drying red oak axes resulted in a free radical signal which was 6-fold larger than that observed in fresh material (Table 3.1). When second derivative scans were run (Fig 3.3B), a second peak could be seen to the right of the main peak (the high field, low-g side). Similar signals were obtained using horse chestnut (Figs 3.4A and B) and *Q. robur* samples (Fig 3.5B), however in horse chestnut the signal increase was marginally smaller (four-fold) upon desiccation. The LF and HF g-values were found to be 2.0040 ± 0.005 and 2.0015 ± 0.015, 2.0060 ± 0.015 and 2.0020 ± 0.005 and 2.0051 ± 0.005 and 2.0026 ± 0.001 (Goodman *et* al., 1995), respectively, for the embryonic axes of red oak, horse chestnut and pendunculate oak. These signals at low moisture showed the same overall shape as those recorded by Hendry *et al.*,

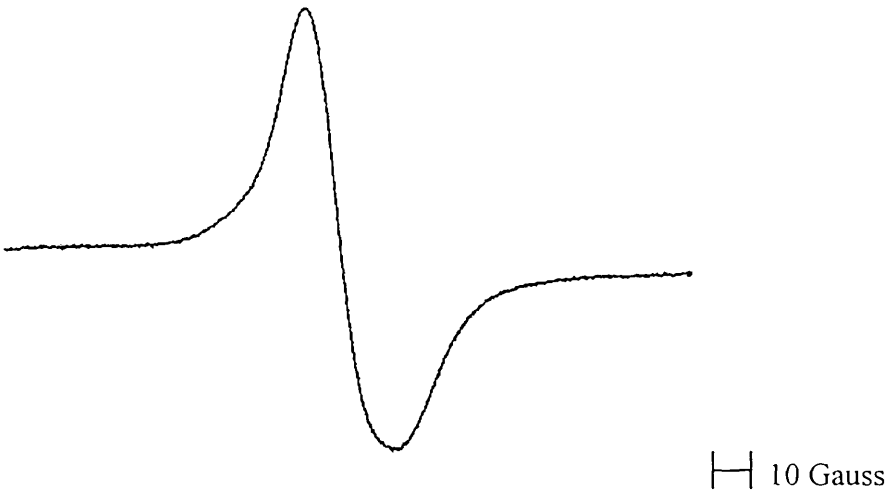


Figure 3.3A

First derivative EPR spectra showing the single peak signals derived from embryonic axes of red oak (*Quercus rubra* L.) using the rapid freeze (-196 °C) technique (receiver gain = 1.0e + 06). Embryo moisture content was 59.8 ± 0.1 %

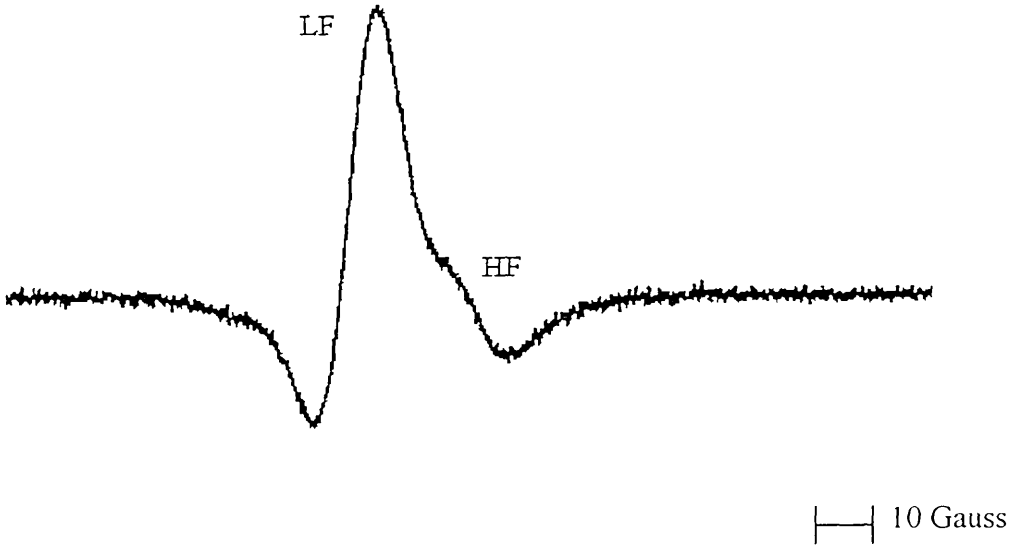


Figure 3.3B

Second derivative EPR spectra detailing the twin peaked signal, showing the low (LF) and high field (HF) peaks derived from embryonic axes of red oak (*Quercus rubra* L.) using the rapid-freeze (-196 °C) technique (receiver gain = 2.5e + 05). Embryo moisture content as above.

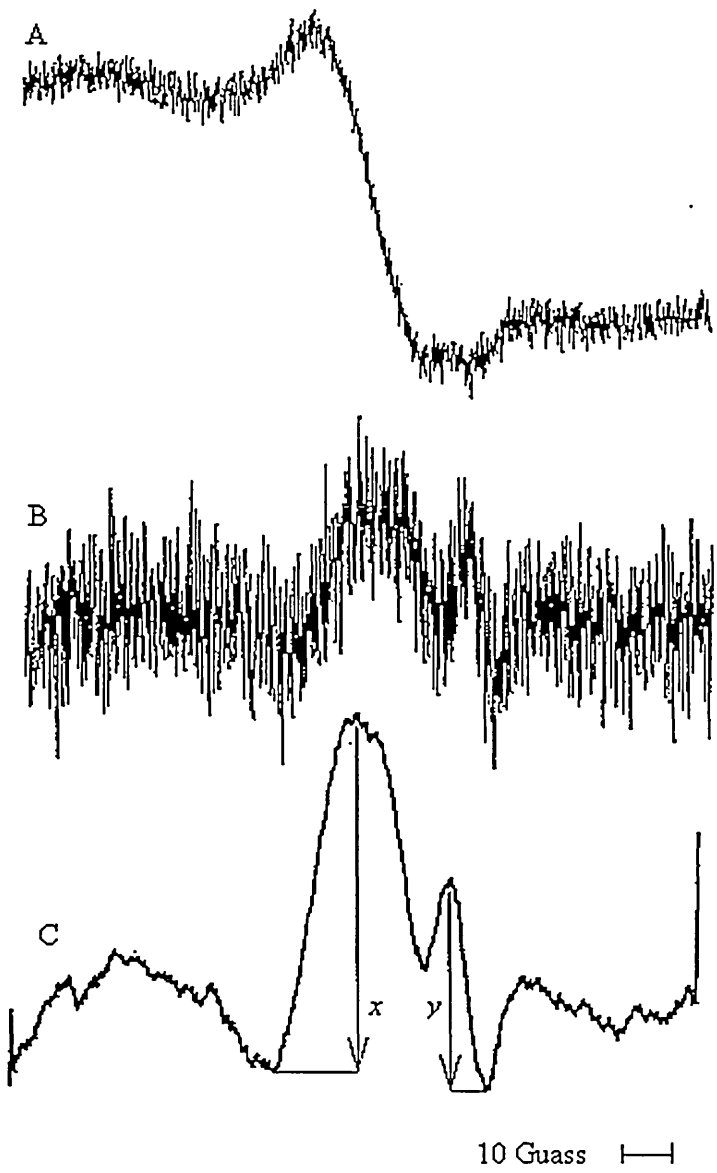


Figure 3.4

Representative EPR spectra from desiccated *A. hippocastanum* L. seed embryonic axes (moisture content 23.0 ± 6.0 %). (A) showing first derivative spectra and the single peak signal obtained (receiver gain = 1.0e + 06 Guass), (B) shows second derivative spectra (receiver gain = 2.5e + 05), the increased resolution enables two free radical peaks to be identified. A smoothed second derivative spectra (C) shows that the signal consists of both a (*x*) low field (LF; high-g) and a (*y*) high field (HF; low-g) signal. Vertical arrows represent the measurement of deflection recorded for each free radical species.

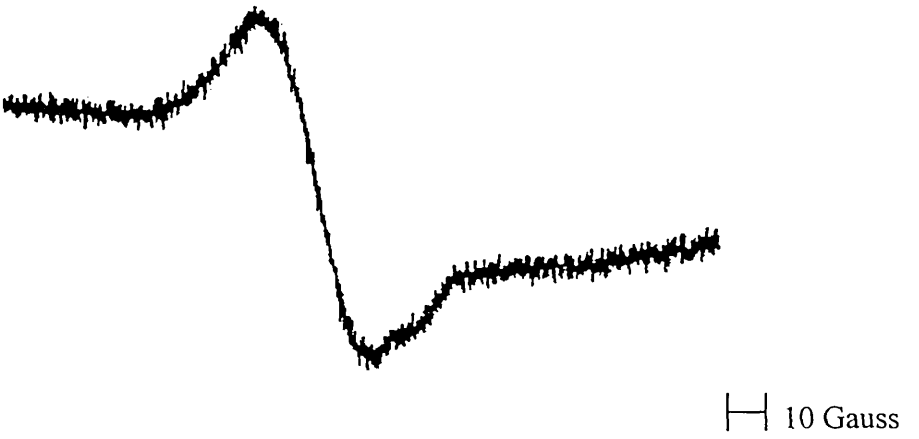


Figure 3.5A

First derivative EPR spectra showing the single peak signal derived from embryonic axes of English oak (*Quercus robur* L.) seed using the rapid-freeze (-196 °C) technique (receiver gain = 1.0e + 06). Embryo moisture content was 52.9 ± 0.6 %.

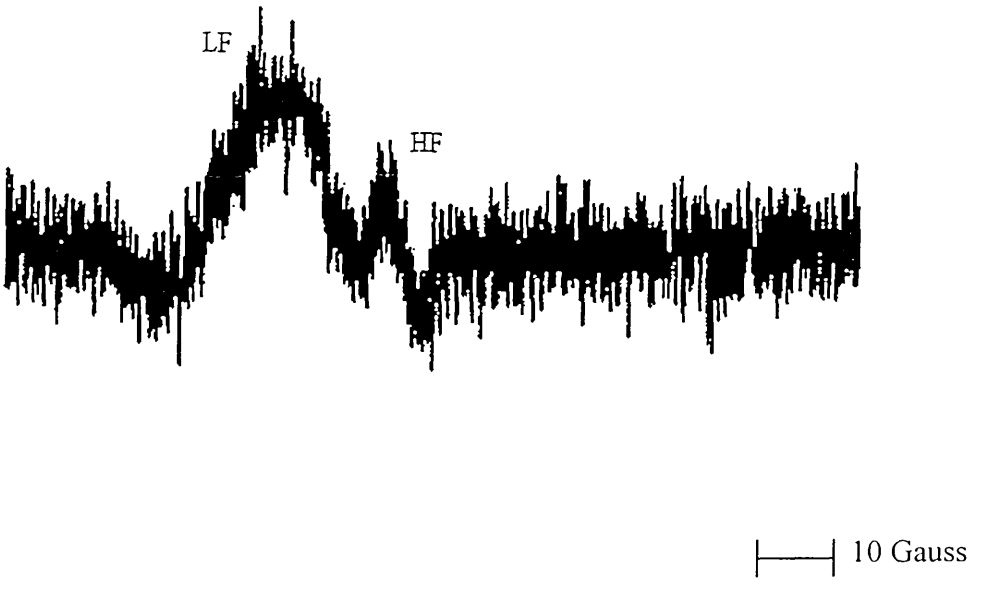


Figure 3.5B

Second derivative EPR spectra detailing the twin peaked signal, showing the low (LF) and high field (HF) peaks derived from embryonic axes of English oak (*Quercus robur* L.) axes using the rapid-freeze (-196 °C) technique (receiver gain = 2.5e + 05). Embryo moisture content as above.

Table 3.1

A comparison of EPR free radical measurements, conducted at -196 °C for both low field (LF) and high field (HF) signals obtained from second derivative scans of embryonic axis tissue of Aesculus hippocastanum *L.*, Quercus robur *L. and* Quercus rubra *L. Seeds were desiccated for upto 80 h, resulting in the moisture content ranges shown.*

Species	Moisture content (%)	LF peak signal intensity (mm deflection)	HF peak signal intensity (mm deflection)
Aesculus hippocastanum L.	64.71 ± 0.71	19.244	12.448
	24.07 ± 1.05	37.432	14.780
	14.19 ± 0.58	49.434	9.202
Quercus robur L.	50.78 ± 1.46	26.328	13.456
	8.87 ± 0.83	129.34	-
	5.33 ± 0.17	168.286	16.678
Quercus rubra L.	50.78 ± 5.78	29.996	14.474
	10.23 ± 4.33	133.452	-
	7.23 ± 0.38	157.968	17.586

(1992) who also investigated free radical activity in pendunculate oak seed components.

3.3.3 Effects of tissue quality and pigmentation upon EPR signal intensity

The seed lot of pendunculate oak used for the investigation of necrosis was 38 ± 1 % viable (n = 2 x 15; seeds sown at 16 °C in the dark). An EPR investigation of the axes revealed that necrosis affected the signal intensity (Fig 3.6). Measurements conducted at ambient temperatures revealed that no signal was detectable in healthy material, or that which was 50 % necrotic (Fig 3.6A and B). However, a free radical signal was evident in totally necrotic material (Fig 3.6C). When the experiment was repeated at -196 °C using the same material, the effect of necrosis was more clear. Again the healthy material showed only a small free radical signal (Fig 3.6D), but the signal increased sharply when the material was 50 % (Fig 3.6E) and fully necrotic (Fig 3.6F).

When necrotic areas were dissected from the embryonic axes that were 50 % necrotic, the signal intensity fell to that just above normal healthy tissue, when measured at -196 °C. This result demonstrates that the necrotic areas were primarily responsible for the increased free radical signal. The slight increase in signal intensity seen upon dissection possibly reflects oxidation caused by membrane disruption, during the multiple dissection of necrotic areas.

Any form of tissue discolouration was therefore expected to influence the free radical signal. To investigate this further, the effects of pigmented tissue on the free radical

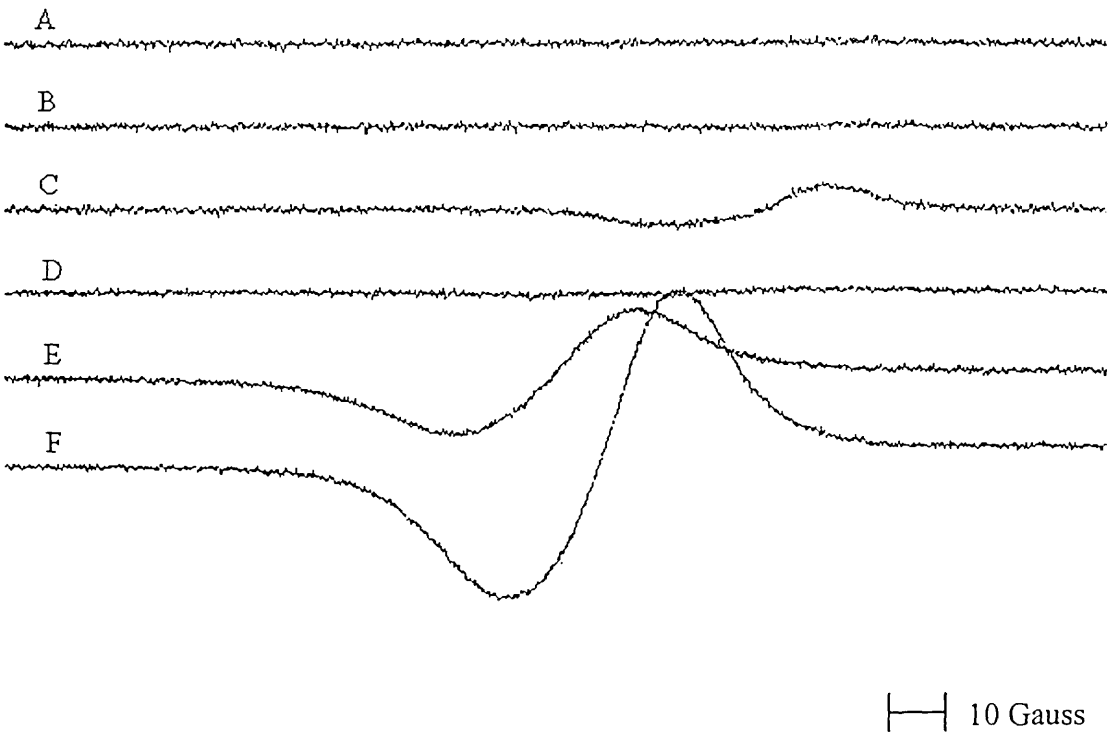


Figure 3.6

Averaged first derivative EPR spectra from 3 runs showing the single peak signals derived from English oak (*Quercus robur* L.) embryonic axes, suffering from various stages of necrosis. The top three traces were conducted at 20 °C, using the surviving tissue technique, and represent 0 (A), 50 (B) and 100 % (C) necrosis. The bottom three traces were conducted at -196 °C using the rapid-freeze technique, again representing 0 (D), 50 (E) and 100 % (F) necrosis (receiver gain = 2.5e + 05).

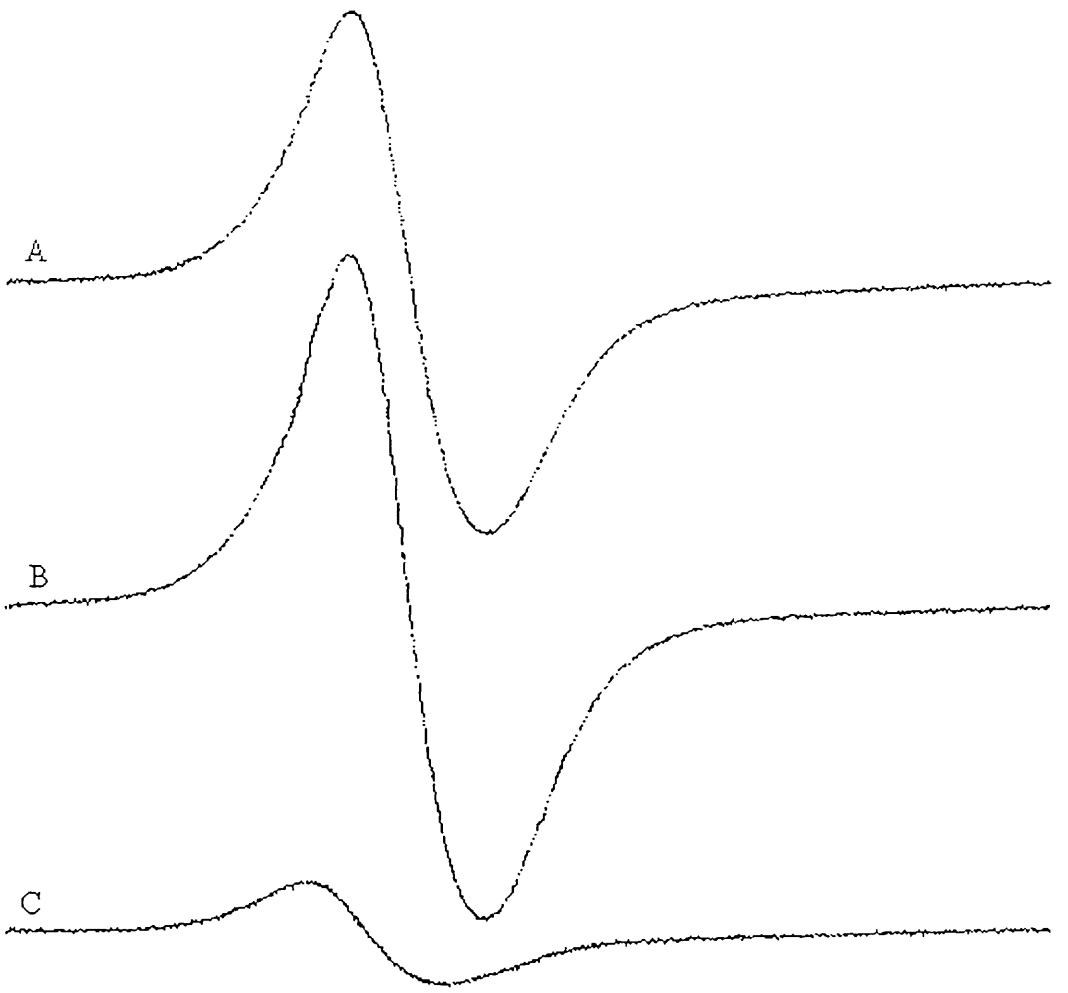
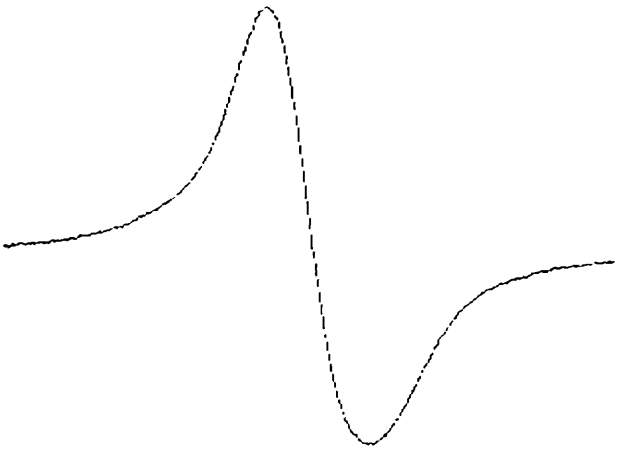


Figure 3.7

Averaged first derivative EPR spectra from 3 runs, showing the single peak signals derived from neem seed (*Azadirachta indica* L.). Traces represent the whole stone (A), the seed (embryo with surrounding mesotesta); (B) and the 'naked' embryo with mesotesta removed (C). Moisture content was 14.3 ± 0.9 %. All measurements were conducted at -196 °C using the rapid-freeze technique (receiver gain = 2.5e+05)



10 Gauss

Figure 3.8A

Averaged first derivative EPR spectra from 3 runs, showing the single peak signal derived from paw paw (*Carica papaya* L.) seed testas. Moisture content was 68.6 ± 5.6 %. All measurements were conducted at -196 °C using the rapid-freeze technique (receiver gain = 1.0e + 06).

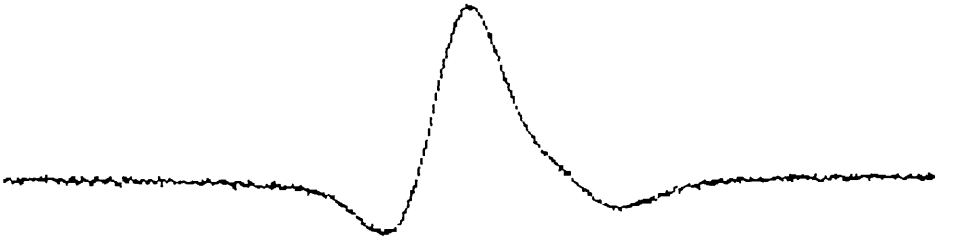
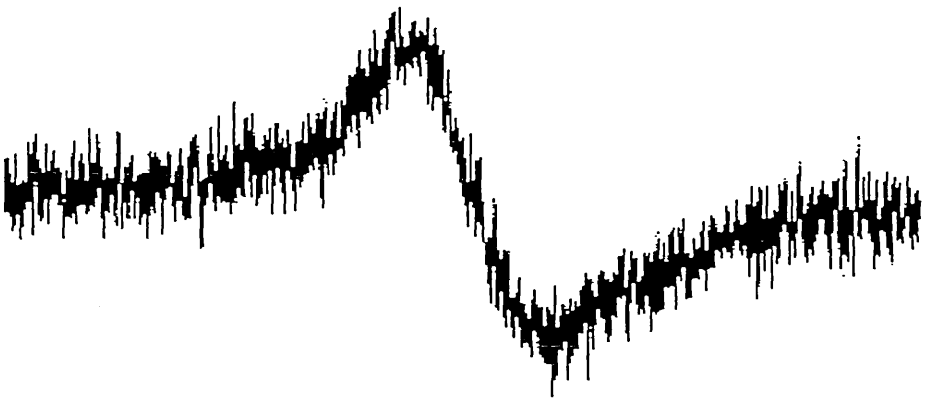


Figure 3.8B 10 Guass

Averaged second derivative EPR spectra from 3 runs, showing the single peak signal derived from paw paw (*Carica papaya* L.) seed testas. Moisture content was as above. All measurments were conducted at -196 °C using the rapid-freeze technique (receiver gain = 2.5e + 05).



10 Gauss

Figure 3.9A

Averaged first derivative EPR spectra from 3 runs, showing the single peak signal derived from 'naked' paw paw (*Carica papaya* L.) seed (testa removed). Moisture content was 49.6 ± 2.4 %. All measurements were conducted at -196 °C using the rapid-freeze technique (receiver gain = 1.0e + 06).

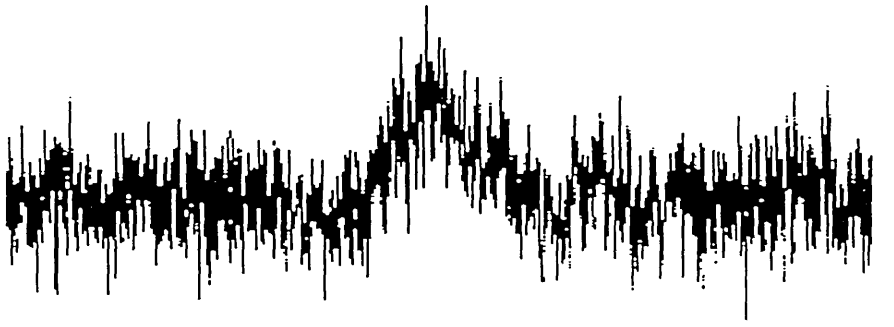


Figure 3.9B 10 Guass

Averaged second derivative EPR spectra from 3 runs, suggesting a two peaked signal derived from 'naked' paw paw (*Carica papaya* L.) seed (testa removed). Moisture content was as above. All measurements were conducted at -196 °C using the rapid-freeze technique (receiver gain = 2.5e + 05).

signal were investigated in neem. Fig 3.7 shows the spectra obtained from neem seed tissues. First derivative scans of the whole seed showed a very large signal intensity (Fig 3.7A). Removal of the endocarp to reveal the seed had little effect on signal intensity (fig 3.7B). However, when the mesotesta was removed signal intensity was reduced c. 6-fold (Fig 3.7C). Figs 3.8 and 3.9 show spectra obtained from papaya seed. First derivative scans of the whole seed again show a very large signal intensity (Fig 3.8A), second derivative scanning indicates that the signal comprises of a single peak (Fig 3.8B). However, when the testa is removed the signal is much reduced (Figs 3.9A and B). In common with other material investigated the 'naked' papaya seed suggests a multi-peaked signal upon second derivative analysis.

3.4 Discussion

It is clear then, that when techniques are applied for the first time to oxidative investigations in which they have not been routinely used, it is important to determine their functionality, to ensure that the method is valid. Therefore, the analytical aspects of the project were thoroughly validated before they were used routinely. This involved examining various aspects of both EPR measurement (this Chapter) and the biochemical assays techniques, that were to be used during the study (Chapter 4). It was important to establish that the results obtained from experimental EPR investigations accurately reflected the biophysical events occurring within the seed material during drying, and were *not* subject to the technical limitations of the method employed, or the physiological status of the seed

/ embryo (e.g. pathology, biochemical profile in relation to pro-oxidative secondary product components). Here I have shown that numerous factors can influence the biophysical detection of oxidative chemistry in seeds, these will be discussed in detail as follows:

3.4.1 Modelling the EPR signal intensity response in relation to water content and temperature

It is widely documented that water can affect EPR signal intensity (for a further discussion see Knowles *et al.*, 1976 or Chapter 7). This is because in hydrated material microwave energy is absorbed by 'free' (unbound; type III) water with a concomitant loss in free radical signal intensity. A convenient analogy can be found with the domestic microwave oven, here the electromagnetic radiation is used to vibrate water molecules present in food. It is the high resonant frequency of the water molecules which warms the food. They vibrate to a such high extent because water is very effective at absorbing microwave energy. In the cavity of an EPR spectrometer water also absorbs the incoming microwave radiation, thereby reducing both the energy available for the resonance condition and the subsequent level of intensity of signal.

The discussion of how water contents can affect free radical determination by EPR, also has a fair amount of experimental evidence to support it. Randolph *et al.*, showed as early as 1968 that EPR spectral intensity obtained from seeds of *Allium cepa* depended upon the seeds water content. Their paper describes a type of water quenching effect on EPR signal intensity when measurements are conducted at

ambient temperatures, and go as far as warning other workers as to the dangers of misinterpreting background water effects. They also suggest that it is important to demonstrate that the effects of moisture *per se* are insignificant before the confident evaluation of EPR signals can be achieved in hydrated seed material.

This raises the issue of whether the contrasting results reported in seed storage biology in the past may have resulted from differences in the temperatures used for EPR measurement by various research groups.

The effect of measurement temperature is of course directly related to the level of seed hydration. Priestly *et* al., (1985) demonstrated this effect in endospermic fractions of *Zea mays* and *Typha latifolia*, where free radical activity declined in all tissues as it hydrated above 7 % moisture content. This may relate to the idea that there are distinct types of water found in plant material (Haber and Randolph 1967, Seewalot *et al.*, 1981, Vertucci and Leopold, 1984, Priestly *et al.*, 1985 and Vertucci and Farrant, 1995). Below c. 7 % moisture content (f. wt. basis) water is bound very tightly to macromolecular structures (type I), and is difficult to remove due to its high tissue affinity. Above 7 % water is less tightly bound (type II) and above c. 20 % free water (type III) is present. Priestly *et al.*, (1985) have suggested that the loss of free radical signal, and hence free radical stabilisation, corresponds to high hydration levels and loosely bound water. Such studies are supported by the findings of Boveris *et al.*, (1980, 1983 and 1984) where chemiluminescence emissions also varied during differing stages of imbibition. This area of research is continuing using the latest advances in technology. Recent studies have involved the use of nuclear

magnetic resonance (NMR) micro-imaging to 'see' water movement within imbibing and desiccating seeds (Pritchard and Glidewell, pers. com.).

Similarly, my studies on the model system DPPH showed little or no free radical signal in wet samples measured using EPR at 20 °C, and a large increase at lower moisture levels. However, the stability of the signal at -196 °C, irrespective of the moisture level, indicates that the former result(s) artificially relate to determinations being made at 20 °C. Thus, there is a physical effect of water removal on the EPR free radical signal intensity when determinations are made at, or close to, room temperature, where water exists in the liquid state.

Rapid-freezing in liquid nitrogen overcomes the difficulty of high water contents by 'freezing-out' water as ice, which is more-or-less transparent to microwave energy (Knowles *et al.*, 1976). I contend that the use of this technique or a similar one *may be* essential for observing the signal obtained from paramagnetic species, in wet or partially hydrated seed tissue.

Importantly I demonstrate that the free radical 'quenching' effect of water at room temperature (Fig 3.1), can be alleviated by measurement at -196 °C (Fig 3.2). The results from the DPPH experiments described earlier employed both rapid-freezing and surviving-tissue techniques (see section 2.3.1.1), and demonstrated two main things concerning hydration levels; firstly that water can have a 'quenching' effect on signal intensity if spectra are recorded at ambient temperatures, and secondly that this effect can be alleviated by conducting the experiments at sub-zero temperatures

using a rapid-freeze technique. When the spectra were recorded at ambient temperatures the reduction in signal amplitude intensity (see Fig 3.1; not to scale) was approximately 63-fold (from 95 to between 1 to 2 mm deflection). It is disturbing to note that some recent investigations of the effects of desiccation on seed material report similar changes in signal intensity responses upon drying, when measured at ambient temperatures (Hendry *et al.*, 1992 and Finch-Savage *et al.*, 1994).

It seems therefore, that when EPR measurements are conducted at ambient temperatures large increases in free radical signal at lower moisture contents could be apparent, simply because the free radical signal is being 'quenched' by type III water at high moisture contents. When type III water is removed during desiccation, this 'quenching' effect is lost, and the EPR signal intensity is seen to increase. Any biological interpretation of this phenomenon would be false. However, when a rapid-freeze method is employed, the frozen type III water is transparent to the incoming microwave energy, and the signal remains strong. The use of this approach is recommended for future studies in the hope that it will alleviate some of the ambiguities of seed hydration level that have plagued so many EPR investigations in the past.

EPR measurements conducted at ambient temperature can also suffer from another problem, which stems from the fact that microwave energy can cause localised heating of tissue (akin to a domestic microwave oven). Plate 3.1 shows the results of power scans that were performed at 20 °C, the pendunculate oak embryonic axis at

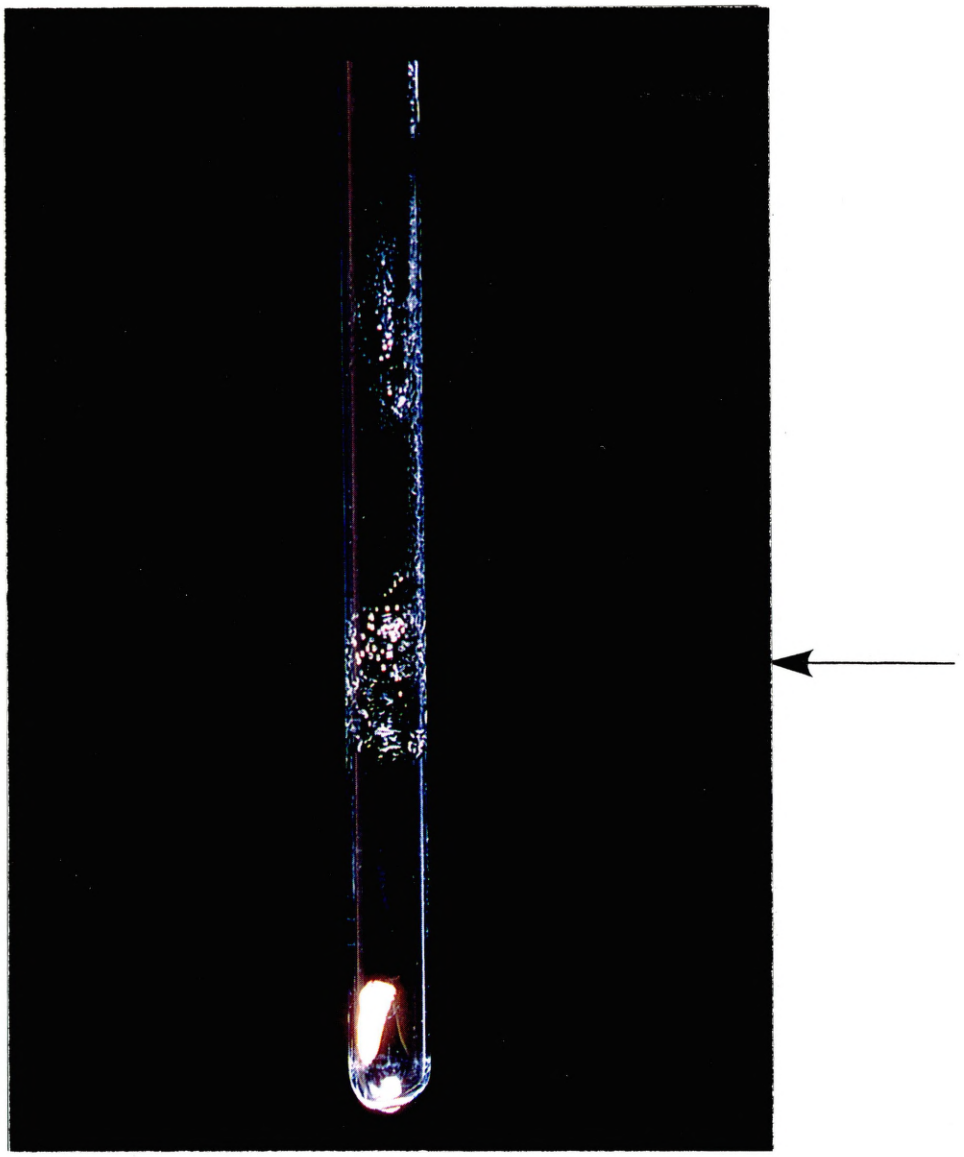


Plate 3.1

The effect of repeated power scanning (using a varying microwave power regime) for 25 min at 20 °C on an embryonic axis of pendunculate oak. Tissue has suffered localised heating and water lost has re-condensed at the point arrowed on the walls of the closed Suprasil tube, concomitant with the zone of incidental microwave energy.

the bottom of the Suprasil tube, has been subjected to a varying microwave power regime for 25 mins (with the power increasing by 0.5 μW every 5 min from 10.5 to 13 μW). The tissue has clearly suffered from localised heating, and water has evaporated from the sample. It has then re-condensed on the walls of the closed Suprasil tube just above the zone of incident microwave energy. For seed material that requires several repeat scans (for example that suffering from a low signal : noise ratio), there is a possibility of not only locally heating the material, but also partially desiccating the material during measurement.

3.4.2 Does the origin of the seed material affect it's EPR signal intensity ?

Another important consideration when studying biological material is it's origin, and function, as these may have important implications upon analysis. It has been well reported that material from different parts of the seed can display levels of metabolic and cellular activity that are dissimilar (see section 3.3.2 and Chapter 4). In this study, the embryonic axes of seeds also tended to show higher free radical content than did the rest of the seed (see Table 3.2). Moreover, both Hendry *et al.*, (1992) , and Finch-Savage *et al.*, (1994 and 1996) have found that free radical, and lipid peroxidation activity were more prevalent in the embryonic axis than other parts of the seed using similar material. Indeed, earlier studies by Seneratna *et al.*, (1988) using purified microsomal extracts, eliminated the effects of bulk changes occurring in other tissues. Such changes may mask those occurring within more sensitive tissues (e.g. the embryonic axis), and therefore measurement at a component tissue level gives a more sensitive test for oxidative stress.

Table 3.2

A comparison of EPR measurements, conducted at -196 °C, showing the difference between low field (LF) signal intensity values taken from second derivative scans of embryonic axis and cotyledonary tissue of Aesculus Hippocastanum *L.*, Quercus robur *L. and* Quercus rubra *L. seeds.*

Species	Moisture content (%)	LF peak signal intensity (mm) for the embryonic axis	LF peak signal intensity (mm) for the cotyledons
Aesculus hippocastanum L.	**64.71 ± 0.71**	**19.224**	**2.005**
Quercus robur L.	**50.78 ± 1.46**	**26.328**	**6.180**
Quercus rubra L.	**50.78 ± 5.78**	**29.996**	**5.346**

The findings that horse chestnut, pendunculate oak and red oak embryonic axes produced larger free radical signals after desiccation (Table 3.1) suggests that oxidative stress may be associated with desiccation intolerance. Such stress may derive from more than one free radical species. Second derivative scans used to enhance signal resolution in seed material (where the signal can be somewhat shrouded by background 'noise') revealed the possible presence of more than one free radical species. Spectra consisted of two overlapping peaks (a high- and low g-peak) in all species. A detailed account of how desiccation influences these peaks is given later (Chapter 5).

3.4.4 Effects of tissue quality and pigmentation on EPR determination

Pendunculate oak seeds of lower overall quality than those used for the desiccation intolerance investigations on recalcitrant seeds, exhibited quite severe necrosis. This

is observed as a blackening of tissue, mainly due to the build-up of polyphenolic compounds within senescent cells (Leshem, 1988).

The point at which the seed succumbs to necrosis depends ultimately upon how much of the seed has become necrotic. If, the embryonic axis is totally necrotic the seed will fail to germinate, and germination can be poor or brief when a large percentage of the axes or cotyledonary material is affected. EPR investigations revealed that necrotic lesions gave large free radical signals. This is obviously of concern if the technique is to be used routinely with seed material. However, this finding does not reveal the source of the increase in EPR signal intensity.

It is known that free radicals are produced during the degradative processes occurring within senescent tissue (Leshem, 1988), but there may be other reasons for the increase in signal intensity that occurs in necrotic material. Chemicals that are deeply pigmented (i.e. have intense UV / visible absorption spectra) very often contain unpaired electrons (and hence constitute free radicals or transition metal ions). Necrotic lesions become pigmented due to the build up of polyphenolics. Is it just these chemicals that give rise to increased signal intensity, or can any pigmented material produce a similar effect (e.g. a seed coat for example) ?

The study conducted using neem and papaya seed revealed that pigmentation alone could lead to increases in free radical signal intensity. This finding means that the increases seen in free radical signal intensity in necrotic material do not necessarily relate to the quality of the material used for investigation, but may merely be a result

of the chemical processes involved in tissue decolouration. It does of course mean that the study of highly pigmented seed material needs careful consideration, and evaluation.

To conclude, the use of EPR as a technique to detect free radicals in seed material is of great benefit, it remains the *only* way that free radicals (of sufficient stability) can be directly assessed in such material. However, experimental conditions have to be precisely controlled, and both pre-treatment and storage conditions can affect EPR signal interpretation. Only when all of the factors mentioned above are considered and adhered to can any authoritative conclusion be drawn from using the technique.

It must also be remembered that EPR alone cannot give a full evaluation of oxidative stress, the technique has to be combined with other biochemical measurements. The following chapter, therefore, gives an evaluation of a biochemical study into the oxidative chemistry of a *model* recalcitrant seed species.

3.5 Summary

Desiccation of DPPH at ambient (20 °C) temperatures, between moisture contents of c. 5 to 38 % resulted in a large increase in EPR signal intensity. This could be related to the removal of a 'quenching' effect of type III water on EPR signal intensity upon desiccation. Seed material has also been seen to suffer from localised heating effects over several repeat scans at ambient temperatures. Both of these problems are

overcome by adopting a rapid-freeze process, and conducting EPR measurements under liquid nitrogen (-196 °C).

When the rapid-freeze technique was employed, free radicals were detected in a range of temperate recalcitrant seeds. Axis material showed higher levels of free radical activity than did other parts of the seed. The level of free radical activity was seen to increase upon desiccation within all seed components. The use of second derivative scanning increased the detail of the signal obtained from seed material where background 'noise' affected signal intensity, and showed that more than one free radical species may be present for all seeds examined.

Seed necrosis was also seen to be associated with an increase in free radical signal intensity. However, it was not clear whether this was due to free radical processes occurring within the senescent tissue, or due to tissue decolouration. This has consequences when it comes to measuring pigmented seed material.

CHAPTER 4

Oxidative Stress During Desiccation-Induced Viability Loss in a Temperate Recalcitrant Seed - *Aesculus hippocastanum* L.

Chapter 4 : Oxidative stress during desiccation-induced viability loss in a temperate recalcitrant seed - *Aesculus hippocastanum* L.

Outlay of Chapter

Chapter 4 : Oxidative Stress During Desiccation-induced Viability Loss in a Temperate Recalcitrant Seed - *Aesculus hippocastanum* L.

4.1 Introduction

An accurate evaluation of free radical activity in biological material depends upon several factors, and any assessment needs to deliver results that are accurate and unambiguous. For example the investigation of free radical activity in seeds can be easily achieved through the use of EPR studies, as long as relevant measurement criteria are adhered to (see Chapter 3). However, measurement of free radical activity alone does not give a full evaluation of oxidative stress. The damage that results from free radical activity is often related to the many secondary reactions that ensue, which produce cytotoxic intermediates and breakdown products (Benson, 1990). The consideration of these indirect parameters of secondary oxidative stress are important to the development of a mechanistic understanding of seed desiccation stress.

The extent of oxidative damage that occurs in biological material is also related to its antioxidant capacity, and the study of these protective mechanisms may be revealing with respect to the overall oxidative stress response. Oxidative stress reactions are based on a number of factors including antioxidant status, oxygen tension and hydration, all of which need to be fully considered before a valid investigation of the role of oxidative stress in desiccation intolerance can be undertaken. Ohlrooge and Kernan, (1982) suggested, for instance, that data based on changes in bond

saturation in lipid fractions should be treated with caution, since sensitive membrane components may be in low concentrations in some seeds. Also, lipid peroxidation is dynamic, and although breakdown products can accumulate in the cells, intermediate products can also be degraded or removed enzymatically.

Horse chestnut seed was chosen as the model system to investigate such parameters as a number of features of the seeds physiology have been previously identified. These include desiccation sensitivity, developmental characteristics, and the effects of seed maturation on germination and drying kinetics (Pritchard *et al.*, 1996). The seed is also very easy to handle and germinate, as its germination characteristics have been well studied (Pritchard *et al.*, 1992 and 1996). It is also very easy to dissect out various seed components, enabling the investigation of treatments at a component tissue level.

Desiccation of horse chestnut seeds to c. 30 % moisture content reduces viability to low values (Pritchard *et al.*, 1996). Whether oxidative events contribute to this response remains unresolved. However there is considerable evidence to support an oxidative component to desiccation stress in other recalcitrant seed material (e.g. Dihindsa & Matowe, 1981; Gamble & Burke, 1984; Senaranta & McKersie, 1986; Price & Hendry 1991; Price *et al.*, 1989; Leprince *et al.*, 1990; Dhindsa, 1991; Buckland, Price and Hendry 1991; Hendry *et* al., 1992 and Finch-Savage *et al.*, 1993 and 1994). In contrast, it has also been shown that oxygen is a prerequisite for the maintenance of recalcitrant seed viability (Tompsett, 1983), even over relatively

short periods of time. These contrasting results may suggest a pleitropic effect of oxygen in hydrated plant systems.

In this chapter I report on the oxidative events occurring in the embryonic axes and cotyledons of horse chestnut seeds, during the period of viability-loss caused by desiccation. Damage, due to desiccation stress, was assessed by measurements of lipid peroxidation products (MDA, 4-HNE and TBARS), antioxidant systems (catalase, peroxidase and SOD), respiration rate, electrolyte leakage and the accumulation of stable free radical signals.

4.2 Experimental design

4.2.1 Validation of extraction method and assay techniques

Seed material (c. 0.1 - 0.4 g) was extracted into a clear, non-particulate solution. There are two options when performing such extractions; the seed material can be extracted into an aqueous buffer solution, or into solvents allowing a lipid extraction. The latter technique has been used successfully in lipid peroxidation studies (see Wilson and McDonald, 1986) but requires a separate, sometimes lengthy extraction step. It was better to determine the chemical concentrations of seed material using an aqueous method, as the procedure eliminates the need for a lipid extraction step. The reduction in time taken to then perform the extraction abates the possibility of seed material degradation, and hence possible introduction of errors.

For all assays, standard solutions were made up to relative concentrations by serial dilution using the aqueous phosphate buffer described in Chapter 2. They were then tested following the recommended procedure for each assay (see Chapter 2), in relation to a determined standard curve. Seed extractions were diluted to concentrations within the range of the standard curves (see Appendix I).

4.2.2. Measurement of seed moisture contents and viability

Horse chestnut seeds were freshly isolated in October 1993 (batch 1) and 1994 (batch 2) and were either immediately sampled (controls) or desiccated in the dry-room for varying amounts of time up to and including 80 h. Moisture contents were determined gravimetrically, as previously described (Chapter 2) on seven embryonic axes and remaining seed tissues (testa and cotyledons combined). Seed viability assessment involved directly sowing on 1 % agar-distilled water immediately after the desiccation treatment, followed by incubation at 35 °C in the dark.

4.2.3 Conductivity measurements

Electrolyte leakage measurements were performed on batch 2 seeds. Axes and cotyledon segments (5 x 5 x 5 mm) were dissected from control and dried samples and pre-hydrated at room temperature for 15 min on damp filter paper to avoid imbibitional and / or chilling damage. After soaking, individually, in 12 ml of distilled water, conductivity measurements were taken every 30 min for 3 h.

4.2.4 Measurement of respiratory activity

Rates of respiration were measured using the Gilson submarine single valve differential respirometer as previously described (Chapter 2). Oxygen uptake was determined using three individual embryonic axes, independently of carbon dioxide evolution at 35 ± 1 °C by employing an alkali trap of 10 % KOH to absorb the latter. Oxygen uptake was recorded every 10 mins for 2 h. The system was allowed to openly-vent for 10 min before each experimental run to facilitate equilibration of gas pressure.

4.2.5 Biochemical measurements

Three assays were used to quantitatively determine secondary oxidative stress, in terms of lipid peroxidation and lipid oxidation product formation, as previously described (Chapter 2). These utilised the Bioxytech, S.A. LPO-586 assay kit to determine MDA and 4-HNE concentrations, and a comparative fluorimetric determination of TBARS (Fraga *et* al., 1988). Finally, the total amount of lipid peroxidation was calculated using the Kamiya K-assay (LPO-CC) kit as previously described (Chapter 2). The application of the LPO-586 and LPO-CC kits to seed material was a novel component of the study.

A range of assays were used to determine the antioxidant activity of seed extracts, included were an analysis of peroxidase, catalase and SOD. Measurement of SOD activity was conducted using the Bioxtech, S.A. SOD-525 assay kit, the activity of peroxidase was measured by following the oxidation of guiacol. Catalase activity was recorded by measuring the change in absorbance of hydrogen peroxide.

Antioxidant activity was related to total protein concentrations, using the Coomasie assay technique (all assays were conducted as previously described; chapter 2). Again the application of the SOD-525 kit to seed material is unique to this study.

Finally, to complement the biochemical investigations free radical activity was monitored using the Bruker ESP300E X-band spectrophotometer operating at a temperature of -196 °C, and utilising the rapid-freeze technique as previously described. All measurement parameters were as detailed in Chapter 2.

4.3 Results

4.3.1 Validation of biochemical assay kits

Linear relationships were seen between absorbance at 586 nm and both 4-HNE and MDA standard concentrations from 0 to 200 μm, similarly the concentration of standards of MDA (fluorimetric assay) and BSA (protein assay) were also linear (see Appendix I). Extraction of seed material into phosphate buffer resulted in a clear, non-particulate, aqueous extract. After dilution, spectrophotometric measurements revealed that these fell within the standard concentration ranges for each assay conducted (see Appendix I). The serial dilution of seed extracts also revealed that a linear relationship existed between absorption and concentration, following a typical Beer-Lambert relationship.

A final assessment of assay validity was made by producing absorption spectra for each assay conducted, examples of which are shown in Appendix II. Both standard solutions and seed extracts fell within the area of maximal absorbance, indicating that aqueous extractions were not prone to interference from chemical, or physiological aspects of degradation.

4.3.2 Desiccation

4.3.2.1 *Physiological effects of desiccation*

Initial moisture contents of the embryonic axes were 64.7 ± 0.7 % and 69.0 ± 1.3, and that of the cotyledons 50.6 ± 0.7 % and 51.7 ± 0.8 % for batch 1 and batch 2 seed respectively. During drying these fell to 13.5 ± 0.7 % and 22.6 ± 6.4 % for the axes, and 26.4 ± 1.1 % and 38.4 ± 1.4 % for the cotyledons respectively. Loss of moisture from the seeds was faster in the embryonic axis than the cotyledons (Figs 4.1A and B), but, overall, the drying rate kinetics for each sample were similar (0.14 and 0.16 log moisture content d^{-1} for batches 1 and 2 respectively). Generally, seed viability fell progressively with the decrease in moisture content (Figs 4.2A and B), however, a slight increase was seen initially for batch 2 seeds. The point at which 50 % of viability had been lost (calculated by linear interpolation) was 24 and 38 % moisture content in the axes of seed harvested in years 1993 and 1994 (batch 1 and 2) respectively (Fig 4.2A). Values for the whole seed were 35 and 40 % respectively (Fig 4.2B).

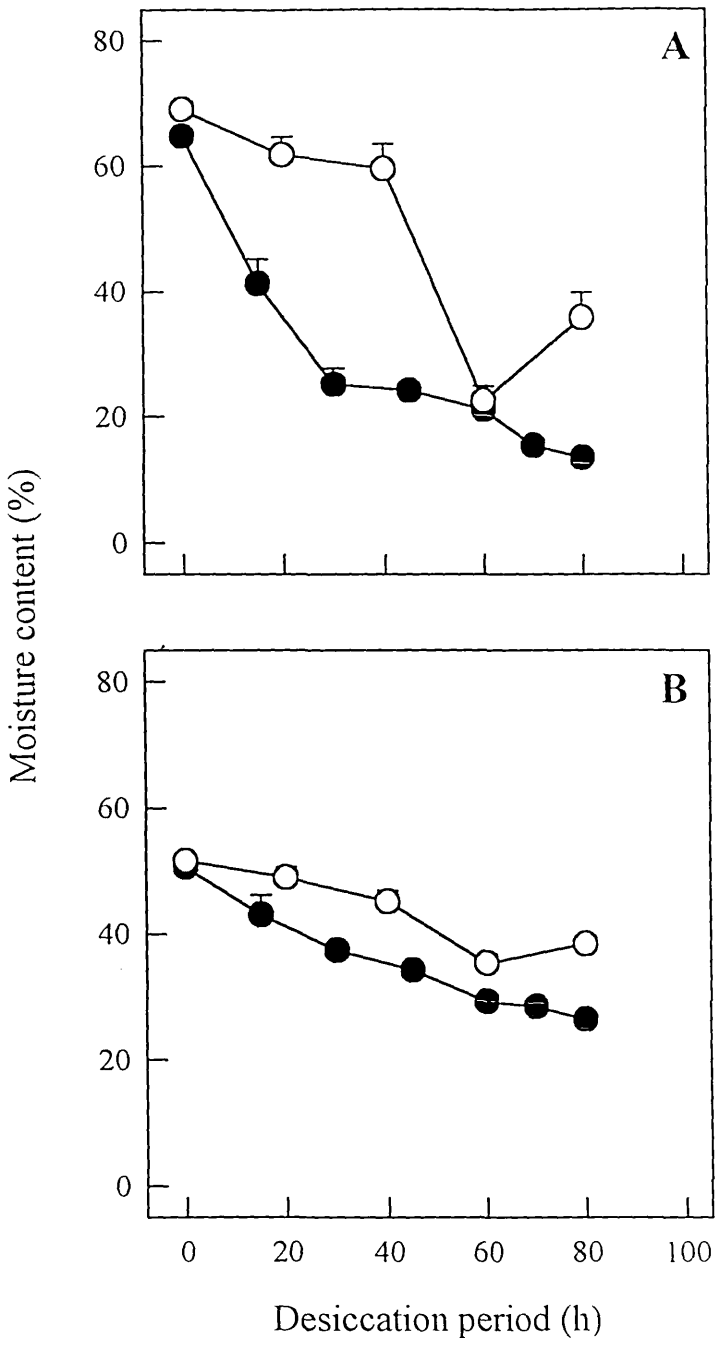


Figure 4.1

Effect of desiccation period on the embryonic axis (A) and the whole seed (B) moisture contents of *A. hippocastanum* L. seed. Seeds were freshly harvested in October 1993 (●) (batch 1) and 1994 (○) (batch 2). Error bars represent one s.d. of the mean.

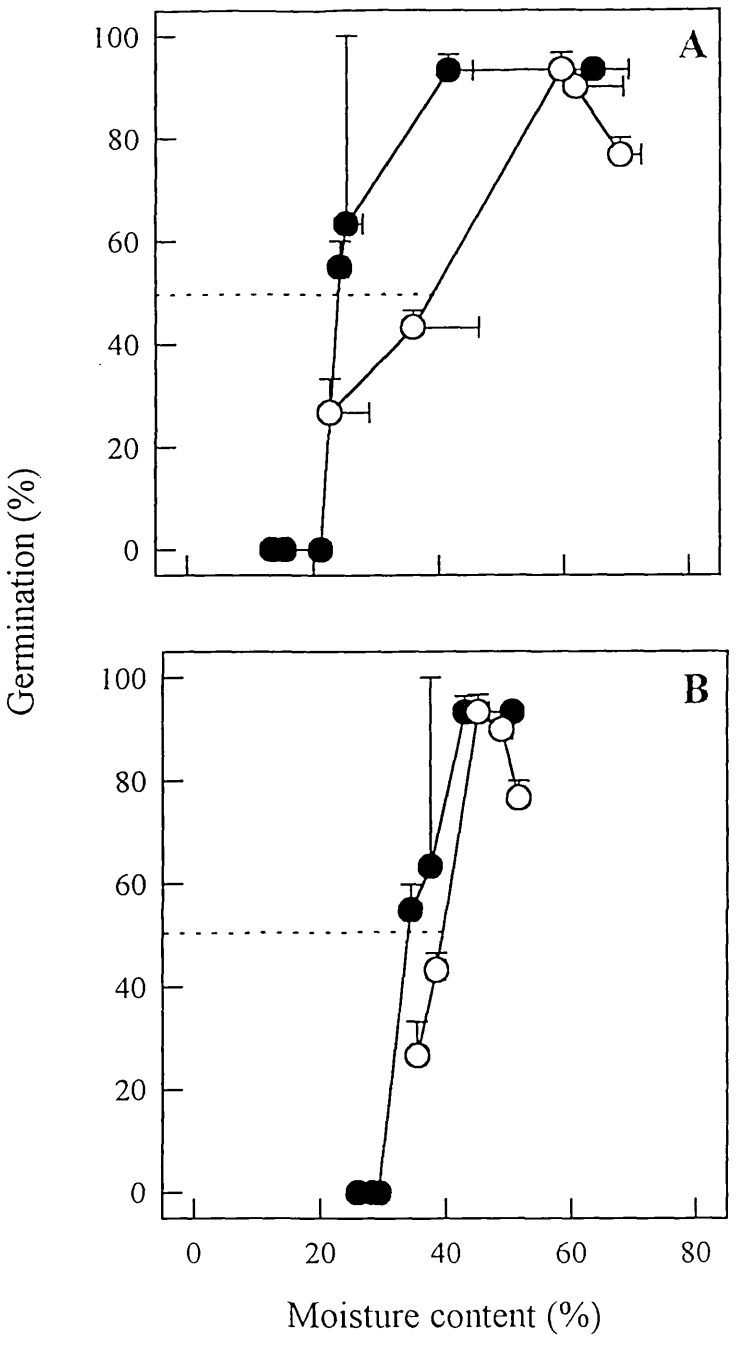


Figure 4.2

Relationship between both embryonic axis (A) and whole seed (B) moisture content and germination of *A. hippocastanum* L. seed. Symbols and bars as Fig 4.1. The dotted line at 50 % germination is included as a measure of desiccation tolerance.

Electrolyte leakage increased upon desiccation in all tissues, but whole seed values were near the limits of detection (data not shown). Leakage was far more prominent in the embryonic axes (Table 4.1) increasing from 9.2 to 46.0 μmhos mg d. wt on drying from c. 69 to 23 % moisture content. Oxygen uptake was almost totally inhibited in seed dried to < 20 % moisture content, a 6-fold decrease compared to freshly harvested material. During the initial stages of drying, respiration rates were fairly constant (up to 1μl mg d. wt min^{-1}), down to a moisture content coincidental with the mid-point for viability loss (Fig 4.2A). However rates then declined to very low levels as the seeds were dried further.

4.3.3.2 *Lipid peroxidation and antioxidant response to desiccation*

Within the cotyledons rises in lipid peroxidation (measured as the accumulation of TBARS) during moisture loss were not significant (r = 0.85; P < 0.05). The lipid peroxidation product profile of the axes following desiccation was seen to be related to viability loss (Figs 4.3 and 4.4). TBARS concentrations in batch 1 embryonic axes only increased upon drying when the axes had reached c. 25 % moisture content. After a peak at about 20 %, further drying resulted in a 30% reduction in concentration (Fig 4.3A). A similar general pattern of response was observed during drying for MDA (Fig 4.3B), 4-HNE (Fig 4.4A) and LPO (Fig 4.4B). For batch 2 seeds the same overall trends were observed. However, increases in lipid peroxidation products were generally smaller, but occurred at similar moisture contents.

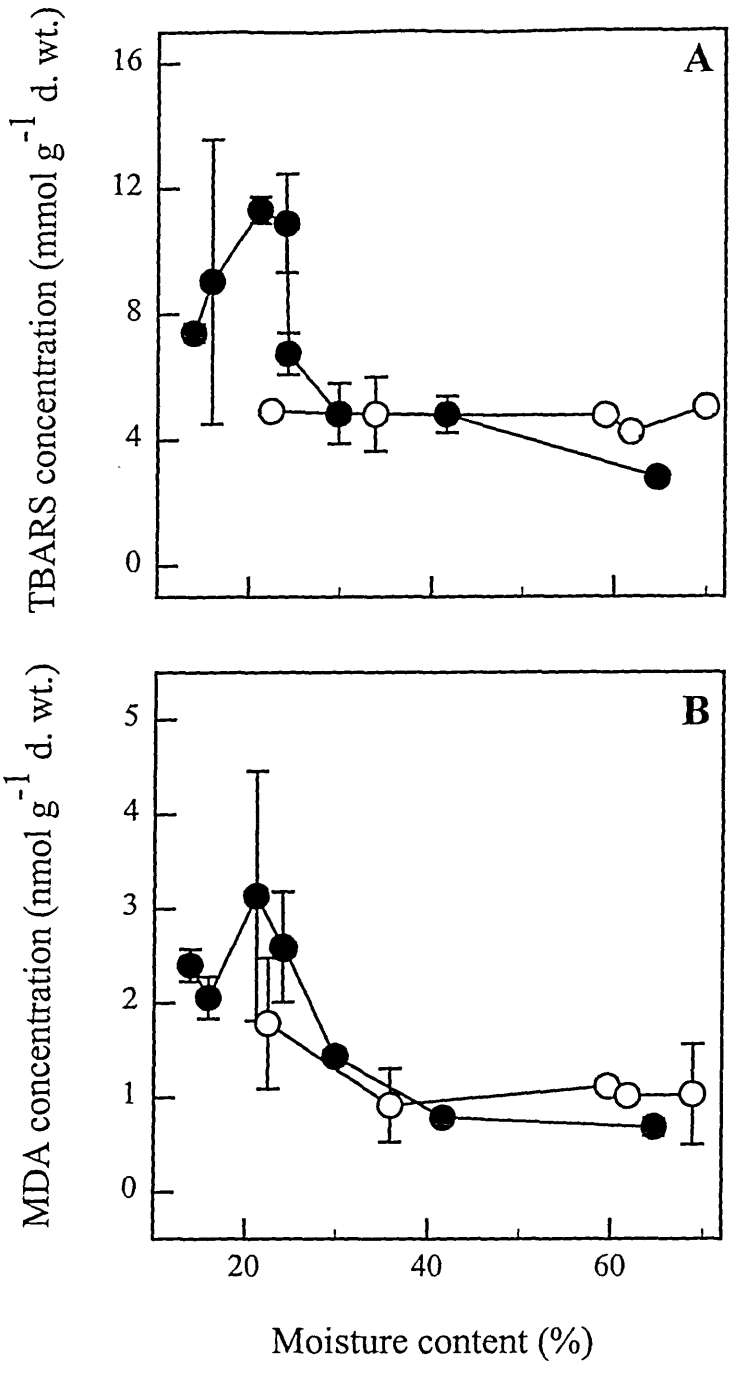


Figure 4.3

Relationship between both the TBARS (A) and the MDA concentrations (B) and the moisture content of *A. hippocastanum* L. seed axes. Symbols are as Fig 4.1 and bars represent s.d.

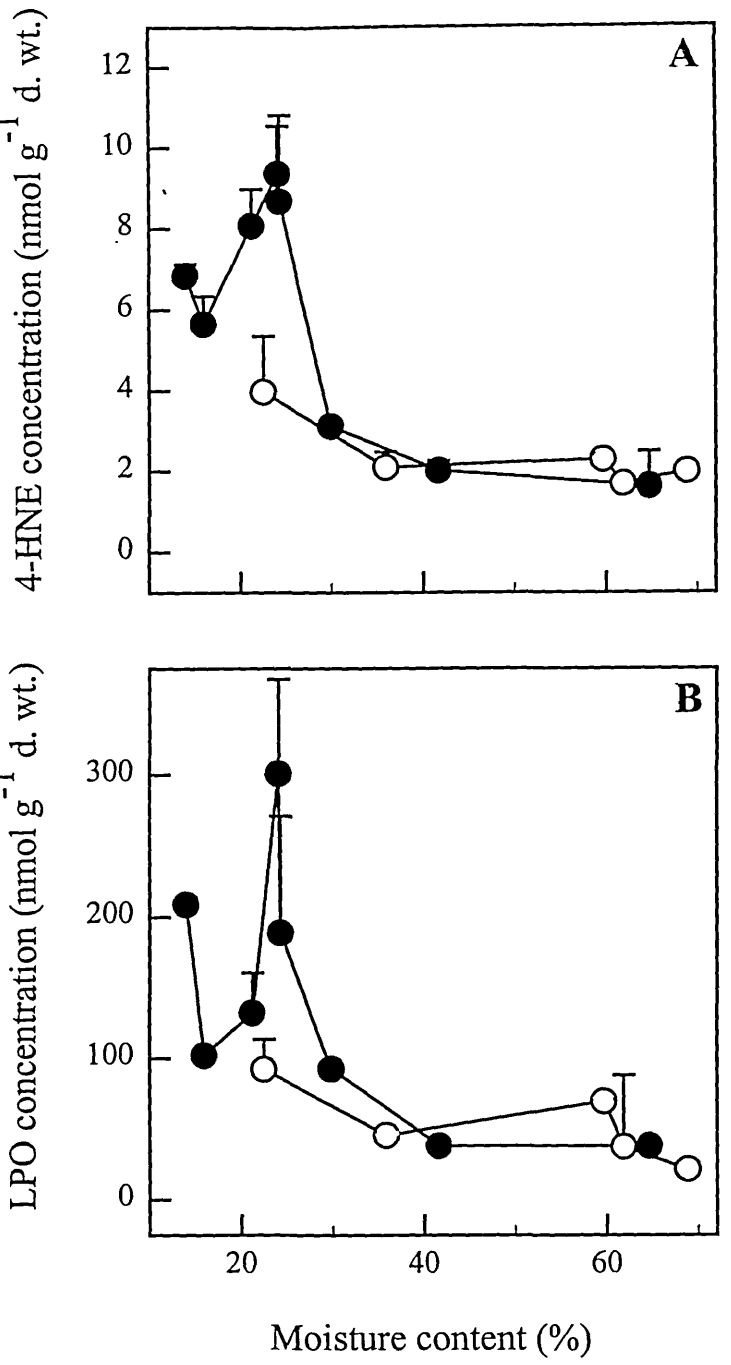


Figure 4.4

Relationship between both the 4-HNE (A) and the LPO concentrations (B) and the moisture content of *A. hippocastanum* L. seed axes. Symbols are as Fig 4.1 and bars represent s.d.

Table 4.1
Effects of drying on the respiratory activity and electrical conductivity of axes extracted from freshly harvested A. hippocastanum *L. seed (batch 2).*

Conductivity and respiration values were recorded after 3 h on three individual axes, which had been rehydrated post-desiccation prior to measurement. For the desiccation experiments on embryonic axis material, data are significantly different ($P < 0.05$) when followed by different symbols.

Axis moisture content after drying (%)	O_2 uptake (µl mg d. wt. min^{-1})	Electrical conductivity (µmhos mg d. wt.)
69 ± 3	117.6 ± 4.6 *	9.2 ± 1.2 *
36 ± 4	126.7 ± 5.7 *	41.0 ± 0.8 +
23 ± 6	17.2 ± 1.8 +	46.0 ± 0.8 #

Table 4.2
Effects of desiccation on protein concentration and antioxidant enzyme activity of axes extracted from freshly harvested A. hippocastanum *L. (batch 2) seed.*

Protein and enzyme values represent the mean ± sd for 3 to 5 axes; triplicate determinations were made on a single extract. For desiccation experiments on embryonic axes, data are significantly different ($P < 0.05$) when followed by different symbols.

Axis moisture content (%)	Protein concentration (mM mg^{-1} f. wt.)	Catalase activity (nmol min^{-1} mg^{-1} protein)	Peroxidase activity (nmol min^{-1} mg^{-1} protein)	SOD activity (SOD-525 units g^{-1} protein)
69 ± 3	1.13 ± 0.03 *	0.12 ± 0.00 *	29.45 ± 9.00 *	1.28 ± 0.26 *
23 ± 6	1.56 ± 0.03 +	0.63 ± 0.00 +	35.00 ± 7.60 *	1.51 ± 0.10 *

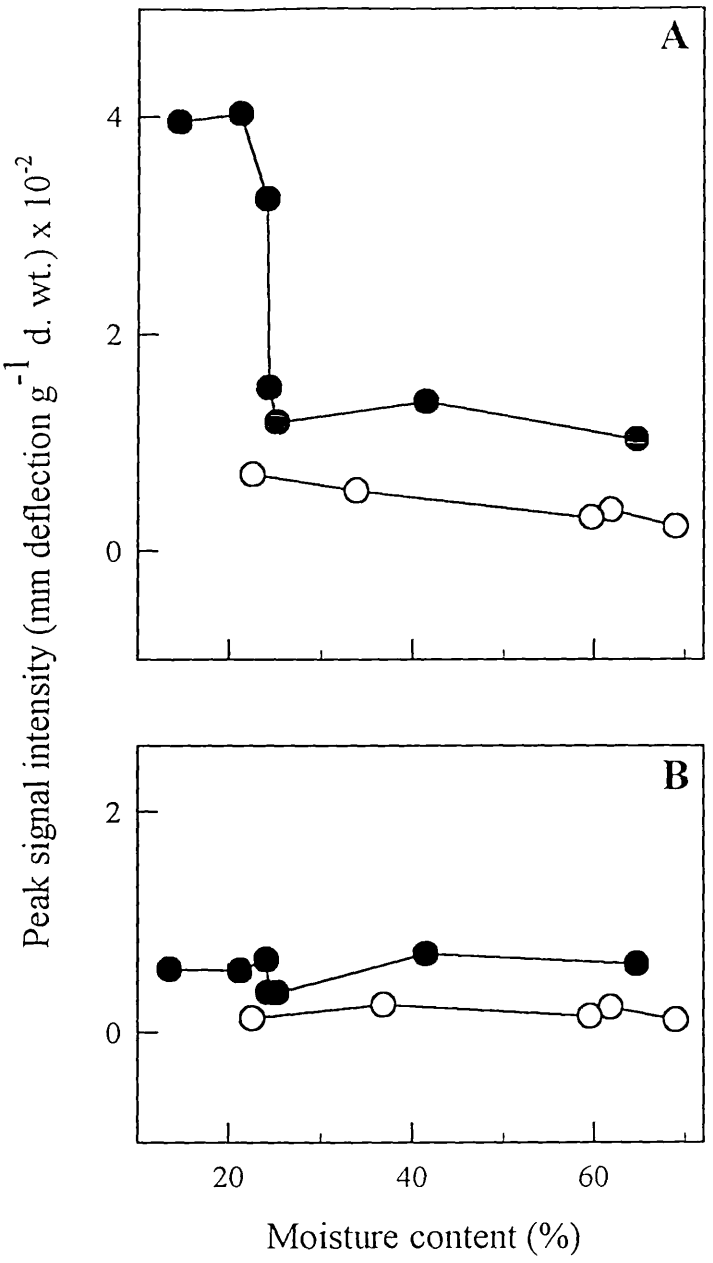


Figure 4.5

Relationship between both the low field (A) and the high field (B) free radical signal intensity and moisture content of *A. hippocastnum* L. seed axes. Symbols are as in Fig 4.1.

The activity of several enzymes involved in the processing of, and protection from, activated forms of oxygen did change; a slight increase in activity of non-substrate specific peroxidase (with guiacol as the reductant) was apparent following water loss (Table 4.2) although this was not significant ($r = 0.85$; $P < 0.05$). Activity of catalase was low in fresh material, but significantly increased upon desiccation. Protein concentrations increased marginally (per g d. wt) upon desiccation. A slight, but non-significant, increase in superoxide dismutase activity was seen following desiccation.

4.3.2.3 *Drying and free radical response to desiccation*

In cotyledonary material free radical accumulation was low compared to that of the embryonic axes (Table 4.3). There was no increase in the LF free radical signal for

Table 4.3
A comparison of EPR measurements for both low field (LF) and high field (HF) signals taken from both embryonic axes and cotyledonary tissue of A. hippocastanum *L. (batch 1) seeds desiccated for up to 75 h.*

Period of desiccation (h)	Embryonic axis		Cotyledonary segments	
	LF peak (mm)	HF peak (mm)	LF peak (mm)	HF peak (mm)
0	62.478	20.298	18.211	5.832
10	41.724	14.184	17.636	8.118
20	61.358	17.557	15.574	6.466
30	50.454	14.465	11.693	6.337
45	44.875	8.168	11.689	4.714
60	108.406	22.515	13.905	5.317
75	47.726	11.373	19.506	10.099

batch 1 embryonic axes early in desiccation until c. 25 % moisture content when there was a four-fold increase (from c. 0.8 to 4 mm deflection g^{-1} d. wt x 10^{-2}; Fig 4.5A). In comparison, there was a gradual increase in batch 2 embryos, this was however significant ($r = 0.95$; $P < 0.01$) and followed the same 4-fold increase trend (from c. 0.2 to 1 mm). No significant increases were observed for the HF signal (Fig 4.5B) in either batch 1 or 2 seeds.

4.4 Discussion

4.4.1 Validation of the biochemical assay kits

Validation of the biochemical assays resulted in standard curves that were within the range specified by the manufacturer, or earlier publications. Dilutions of each seed extraction were constructed to fall within the assays standard concentration range (see Appendix I). These concentrations were determined for each assay, and then used during subsequent investigations.

For a biological assay to deliver a meaningful quantitative result, the extract must adhere to the Beer-Lambert relationship. Colourimetry is based upon the absorption of monochromatic light (either UV or visible) by the solution being investigated, under standard conditions of measurement. The concentrations of ions in the coloured solution are related to absorption by the Beer-Lambert law:

$$\log_e I_0 / I = kcl$$

where I_0 is the incident light, I is the emergent light, c is the concentration, k is a constant (the molecular extinction coefficient) and l is the path length. If concentrations are to be accurately calculated, then a study of absorption values against concentration should be linear (for the ranges specified in the experimental procedures). As the Beer-Lambert relationship was maintained in both standards (Appendix I), and seed extracts for all assays further validation only required the examination of absorption spectra. This was conducted for each assay (see Appendix II), and in each case maximal absorbance / fluorescence coincided with the measurement wavelength of the assay. Further evaluation of the assay techniques using advanced analytical techniques (e.g. HPLC or LC-MS) was beyond the scope of this study, however recent studies have started, using plant material, by other workers (Deighton *et al.*, 1997).

As the extraction into aqueous buffer was successful and serial dilutions fell within the concentration ranges required, this method of extraction was used throughout the investigation. It was convenient and eliminated the need for a separate solvent extraction step, hence reducing the time frame for any possible introduction of errors, and minimising the possibility of metabolic degradation of seed material during the extraction procedure.

The major aldehydes present in horse chestnut seed material were always MDA and 4-HNE, this is in agreement with the work of Esterbauer *et al.*, (1990) who also identified hexanal as a major breakdown product of lipid peroxidation. Esterbauers group worked on human tissue, and other groups (Yoshino *et al.*, 1986 and Selley *et*

al., 1989) have also looked *only* at animal tissue (mainly rat homogenates). To the authors knowledge this is the first time that 4-HNE has been identified in seed tissue, and it is only recently that it has been observed in other plant material; e.g. in plant tissue culture lines (Robertson *et al.*, 1995 and Deighton *et al.*, 1997). 4-HNE is extremely cytotoxic, and is reported to interact with both GSH and protein thiol groups, causing inactivation (Schauenstein *et al.*, 1977). Results presented earlier suggest that 4-HNE mediated inactivation of proteins and an important antioxidant (GSH) may contribute to the loss of viability in desiccating horse chestnut seed (see comment later in this discussion).

4.4.2 The study of desiccation sensitivity

The cause and mechanisms of viability loss in desiccation sensitive (recalcitrant) seeds are not fully resolved. In recent years, many studies have focused on protective components such as late embryogenesis abundant (LEA) proteins (Wechsberg *et al.*, 1994), tissue specific soluble sugars (Steadman *et al.*, 1996) and the stabilisation of oil bodies by oleosin proteins (Leprince *et al.*, 1998). While correlations between chemical composition and seed storage behaviour have been demonstrated in several systems (see Crow *et* al., 1992, Leprince *et al.*, 1993, Vertucci and Farrant, 1995), accumulating evidence also shows that these may not always hold (Blackman *et al.*, 1992, Farrant *et al.*, 1993, Finch-Savage and Blake 1994, Ooms *et al.*, 1994 and Still *et al.*, 1994). An alternative methodology is to study the systems involved in desiccation sensitivity. Here one such system, that of desiccation induced oxidative damage has been critically re-examined using horse chestnut seeds.

4.4.3 Responses to desiccation

A study of the kinetics of horse chestnut seed desiccation revealed that the rapid decrease in moisture content during drying indicates that the testa (seed coat) does not limit moisture loss, and suggests that horse chestnut seeds have no greater ability to retain water, than do other large recalcitrant seeds. It is clear that moisture is lost at a more rapid rate from the embryonic axis, than in the rest of the seed (compare Fig 4.1A to B). This may be due to the position of the embryonic axis in a small radicular pocket directly under the seed coat, where it is more susceptible to air drying (see Fig 4.6A; Pritchard *et al.*, 1996 for morphology). In this species this is an important feature, as this part of the seed undergoes extensive oxidative damage, compared to the rest of the seed. The fact that this sensitive structure, a pre-requisite to germination, is located in an extremely desiccation vulnerable position suggests that the plant may have developed in environments where moisture availability is not a problem during seed maturation and shedding.

Although now common in the U.K., the horse chestnut tree was introduced from Greece (between 1612 - 15; Mitchell, 1996), where rain fall figures range from c. 43 to 95 mm per month during the period of seed maturation and fall (based on data collected from 1932 - 40 and 1950 - 60; HMSO, 1973). Similarly, seed shed in the U.K. coincides with the generally wet early autumn period (October). Clearly, given the ease of removal of moisture from the axis, shedding seeds in the 'wet season' is an appropriate adaptation for a species producing recalcitrant seeds.

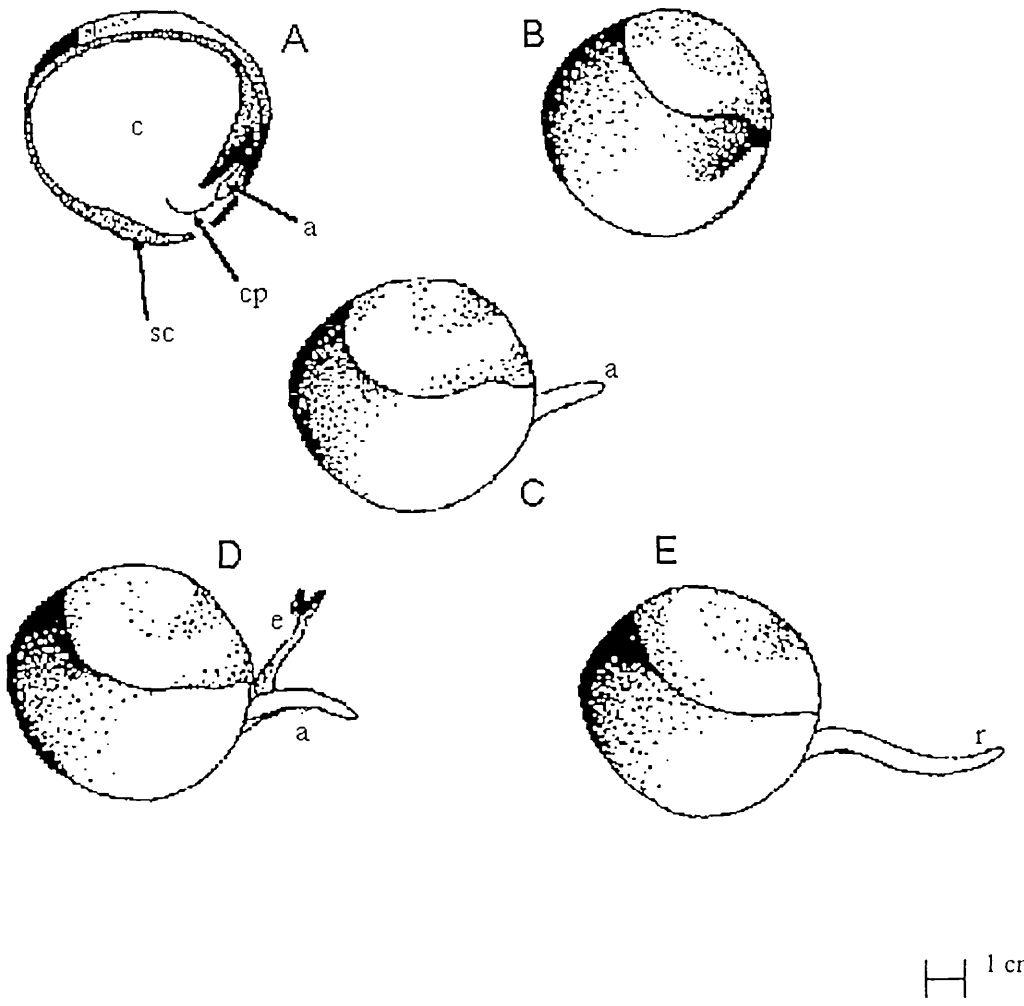


Figure 4.6

Morphology of *A. hippocastanum* L. seed. (A) showing a cross section of a mature seed, as shown in (B), showing the interior of the seed with the seed coat (sc), cotyledonary petioles (cp), cotyledons (c) and embryonic axis (a) arrowed (note the axis sits in a small 'radicular pocket'); the remaining pictures represent different stages of germination; (C) emergence of the axis, which can then be followed by either (D) cessation of growth of the axis, and outgrowth of the epicotly (e) or (E) by immediate rapid growth of the axis through radical extension (r). (Adapted from Pritchard *et al.*, 1996).

The loss in viability of seeds with falling moisture content confirms that horse chestnut seeds are recalcitrant, as reported earlier by Tompsett and Pritchard (1993). Viability loss was observed as moisture contents declined below 40 % and 60 % in the axes of seeds harvested in 1993 and 1994 respectively. In freshly harvested 1994 (batch 2) seed, viability was seen to marginally increase initially upon drying (Fig 4.2A), this may be linked to a maturation effect. Relative improvements in desiccation tolerance have been observed as horse chestnut seeds develop (Tompsett and Pritchard, 1993), and thus the increased desiccation sensitivity in batch 2 seeds and the beneficial germination effects of partial drying suggest that the seeds were collected at a less mature state than batch 1 material (see Tompsett and Pritchard, 1998 for discussion).

Evidence presented in Figs 4.3 to 4.5 and Tables 4.1 to 4.3 (discussed earlier) show that during moisture stress recalcitrant horse chestnut seeds are exposed to activated forms of oxygen, and subsequently accumulate stable free radicals and lipid peroxidation products, which can mediate destructive reactions (see Hendry, 1993, Hendry *et al.*, 1992, Leprince *et al.*, 1990, 1993, 1994, McKersie 1991 and Navari-Izzo *et al.*, 1994). Such changes are consistent with the type of injury observed in the loss of embryogenic potential in vegetative plant tissues in response to other stresses, such as chilling and long term sub-culturing *in vitro* (Benson, 1990). Conversely it has been reported that desiccation-tolerant tissues are capable of evading damage from peroxidative reactions during drying, and a lack of free radical accumulation has also been reported in such material (Magill *et al.*, 1994). Loss of viability in recalcitrant counterparts is associated with various symptoms of free radical injury

such as lipid peroxidation, phospholipid de-esterification and the accumulation of a putative highly stable free radical (Hendry 1993, Hendry *et al.*, 1992, Leprince *et al.*, 1990, 1994 and Navari-Izzo *et al*, 1994). The timing and relative magnitude of oxidative stress responses observed in horse chestnut embryonic axes supports the view that this type of injury is coincidental with viability loss, i.e. it is an early event, following the general sequence:

desiccation → formation and / or exposure to activated oxygen species, including free radical formation (which may initially be extremely transient, but ultimately results in the formation of more stable radicals) → lipid peroxidation → membrane perturbation → damage to proteins (4-HNE and other aldehydics) → viability loss.

This is in agreement with Leprince *et al.*, (1990). However, my results show no indication of events taking place *prior* to viability loss. It has been previously indicated that some precursors to oxidative stress (e.g. jasmonic acid and its methyl ester) may be synthesised *before* cellular death is observed (Finch-Savage *et al.*, 1996), as they may act as a 'hormonal' response, involving cell signalling.

Spectra presented in Chapter 3 and biochemical analyses from this chapter reveal the presence in horse chestnut seeds of a complex free radical signal with both a LF and a HF component. The identification of the free radical components in ageing (senescent) or desiccation stressed material is debatable (Atherton *et al.*, 1993, Goodman *et al.*, 1995 and Atherton *et al*, 1995). It has been suggested that this free radical may arise from desiccation-induced impairment of the electron transport

chain between complex 1 and the ubiquinone pool of mitochondrial membranes, leading to the transient formation of activated oxygen (Leprince *et al.*, 1994). Based on this evidence and on a relationship between increases in respiration rates and desiccation sensitivity (Leprince *et al.*, 1992), respiration has been suggested to play a central role in desiccation intolerance via a free radical mechanism of injury.

The point at which loss of viability was observed during drying, coincided with approximately a quadrupling of free radical signal intensity within the embryonic axes of both batches of seed. The immaturity of batch 2 seed has the unfortunate consequence that this seed lot did not *fully* loose viability upon desiccation (Fig 4.2), hence the lower increase in free radical signal intensity (Fig 4.5). No significant increases in signal intensity were found in the cotyledons. These results follow a trend which Finch-Savage *et al.*, (1994) observed, and suggests that oxidative stress may play an important role in the loss of viability during the desiccation of recalcitrant seed tissues. However, this study departs from earlier reports on free radical behaviour in recalcitrant seeds in two ways, firstly signal amplitude, and secondly the level of detail obtained from spectra.

In regard to with the former issue, previous reports of desiccation induced free radical accumulation in recalcitrant seeds (Hendry *et al.*, 1992 and Finch-Savage *et al.*, 1994) have indicated much larger increases in signal amplitude (from c. 60 to 200-fold). A possible cause of this relates to the quenching effect of type III water in wet seeds on signal intensity when spectra are recorded at ambient temperatures (as discussed in Chapter 3). To alleviate this problem all measurements were conducted

at -196 °C, using a rapid-freeze technique to ensure that all type III water in the tissues was within the solid state, which effectively increases the signal : noise ratio in the spectrometer cavity. Differential scanning calorimetry (DSC) measurements revealed that the % 'un-frozen' water content of desiccated horse chestnut seeds is 20.9 ± 0.7 and 24.8 ± 3.6 (upon cooling) for the embryonic axes and the cotyledons respectively. This supports the hypothesis that type III water is removed during desiccation of horse chestnut seeds, leaving only a small percentage of 'un-frozen' type II / I water remaining.

Secondly, the consequence of recording the spectra of wet samples at ultra-cool temperatures is that in conjunction with second derivative scanning, two peaks could be clearly resolved in wet and dry axis material. A similar resolution of two peaks has only previously been observed in recalcitrant material after partial drying and was not evident in fresh material (Hendry *et al.*, 1992). Although Hendry *et al.*, (1992) did not report g-values for spectra, the measurements made during this study gave g-values of 2.0060 ± 0.015 and 2.0020 ± 0.0005 for the low and high field respectively (Fig 3.4B). These values are comparable to those reported by Atherton *et al.*, (1993) for a desiccated moss, which suggests that biological samples might contain similar free radical components. It is also clear from the studies using horse chestnut embryonic axes (Figs 4.5A and B) that only one of these components (the LF signal) increases upon desiccation, indicating a role for the LF free radical species upon desiccation (see later and Chapters 5 and 6). In contrast, Hendry *et al.*, (1992) assigned particular physiological significance to an increase in the HF-signal on drying, although the LF-signal also increased. The difference in findings between

the two groups is unclear but could relate to the actual identification of the free radical species.

One difficulty in identifying the free radical species involved in the desiccation of recalcitrant seeds is the close proximity of the g-values obtained upon analysis. This makes it very difficult to distinguish between, for example, oxygen- and carbon-based free radicals, when there is an absence of hyperfine structure in the free radical signal (Knowles *et al.*, 1976).

As a result, the identity of the free radical(s) involved in recalcitrant seed desiccation viability loss has been the point of recent speculation (Atherton *et al.*, 1993; Goodman *et al.*, 1995 and Atherton and Hendry, 1995). It has been postulated that the high-g (LF) free radical derives from a quinone. This stems from previous work where the low-g peak was not apparently associated with fully viable seed material, because its absolute concentration was less in samples of high moisture content. Because of the reduced spectrometer resolution this could be an artefact of recording spectra at ambient temperatures. In this study only the high-g (LF) signal increased in intensity upon desiccation, and both signals are still to be identified. This difference in identity of the free radical signal involved in desiccation stress is most probably due to the enhanced sensitivity obtained by conducting measurements at -196 °C, and resolution afforded by second derivative measurement.

It was also very clear that the free radical signal intensities for both peaks were lower in cotyledonary material, suggesting that the embryonic axes had a higher level of

oxidative metabolism than did the cotyledons (Table 4.3). This hypothesis was supported by 'respiratory' data which showed oxygen uptake was easily measurable in the embryonic axes (Table 4.1), but was at or below the limits of detection for the cotyledons (data not shown). During germination the embryonic axis rapidly grows and must be capable of coping with a highly active metabolism. By comparison the cotyledons main function is the slower breakdown of storage material. Not surprisingly then the axes are thought to be a more sensitive marker of stress and, as will be seen in Chapter 5, generally make suitable material on which to make reasonable comparisons of tolerance levels between species.

The increase in free radical signal intensity upon desiccation below certain critical moisture contents was also accompanied by an accumulation of lipid peroxidation products within the embryonic axes, although again, this situation was not as clear for cotyledonary material. I interpret these findings as an indication that stable free radicals formed during desiccation of recalcitrant seeds were products of complex radical events, which included the peroxidative destruction of lipids. An assumption which was supported by significant increases in electrolyte leakage upon desiccation, indicating extensive membrane perturbation.

The subsequent decrease in aldehydic breakdown (lipid peroxidation) products after the point of viability loss may be ascribed to the findings of Ishikawa *et al.*, (1986). When examining rat heart tissue perfused with 4-HNE, these workers found that 4-HNE was very rapidly conjugated to glutathione, the half-life of the 4-HNE being less than 4 seconds. They also demonstrated that the HNE-glutathione conjugate was

transported out of the cells by a carrier-mediated process. The reason for the increased level of conjugate removal in seeds at moisture contents < 20 % may be related to the findings of Gómez-Puyou and Gómez-Puyou (1998). In a recent study of enzyme activity within low water systems they have established that stabilised conformers exist that exhibit unsuspected catalytic properties, as well as intermediates of enzyme function, formation and degradation that in aqueous environments have relatively short life-spans.

The relatively small changes in activity of antioxidants (only catalase increasing significantly upon desiccation; Table 4.2), may indicate the inefficiency of these enzymes to respond to the oxidative events that occurred when horse chestnut seeds were desiccated. If this hypothesis is true, the cytotoxic events occurring during desiccation-induced oxidative stress would overcome the inadequate antioxidant protection available, and a cascade of the destructive oxidative events previously described would ensue, ultimately leading to cell death.

In conclusion, the oxidative responses observed during drying of horse chestnut seeds, imply that free radical mediated events play a significant role in the recalcitrant behaviour of this species. However, in order to establish that this type of response is common to all recalcitrant seeds, an investigation into the oxidative behaviour of seeds covering a wide range of desiccation sensitivities is required. The following chapter therefore describes a study of the oxidative responses to desiccation of a range of tropical recalcitrant seed material, of varying desiccation sensitivity to complement the temperate species already investigated.

4.5 Summary

Aqueous extraction of seed material was successful, and after dilution fell within the range specific for each assay, following a typical Beer-Lambert relationship. Validation of the extraction method and biochemical assays was completed by recording absorption spectra, which were also favourable. Products of lipid peroxidation were found in seed material including MDA and 4-HNE, this is the first study to demonstrate the presence of 4-HNE in seed material.

Desiccation of recalcitrant horse chestnut seeds demonstrated that the seed has little ability to retain type III water, the embryonic axis being particularly susceptible. This may be in part related to the seeds morphology, but was also due to there being a higher oxidative activity in this tissue, a finding that was supported by respiratory measurements. Desiccation resulted in a loss of viability, and the point at which viability was lost coincided with a 4-fold increase in free radical activity within the embryonic axis. The second derivative free radical signal obtained composed of two peaks, but only the LF peak increased upon desiccation.

The increase in free radical activity was accompanied by transient increases in the lipid peroxidation products; MDA, 4-HNE, total lipid peroxides and TBARS. This finding was supported by an increase in electrolyte leakage indicating extensive membrane perturbation. The transient rise in lipid peroxidation products may be a result of their cross-linking or enzymatic degradation upon production. The very

small degree of antioxidant activity observed, upon desiccation, may serve to highlight the inefficiency of these enzymes to counter oxidative stress events, imposed upon horse chestnut seeds when they suffer desiccation stress.

CHAPTER 5

A Comparison of the Oxidative Stress Response of Tropical Seeds with Different Desiccation Tolerances.

Chapter 5: A comparison of the oxidative stress response of tropical seeds with different desiccation tolerances.

Outlay of Chapter

Chapter 5. A Comparison of the Oxidative Stress Response of Tropical Seeds with Different Desiccation Tolerances.

5.1 Introduction

In the previous chapter oxidative events associated with the response of a temperate recalcitrant seed response to desiccation were described; both as increased free radical production and lipid peroxidation. This chapter extends this study to determine if the biochemical profiles already seen in horse chestnut seeds are manifest in desiccation sensitive seeds of other species. Two groups of species have been identified as having seeds which are not orthodox in their behaviour to drying: recalcitrant and intermediate (see Chapter 1 for discussion). Critical levels of desiccation for each type are debatable.

For example, in relation to individual tissues in the seed (Pritchard and Prendergast, 1986 and Finch-Savage, 1992); between species within each group (e.g. papaya; Ellis *et al.*, 1991b and Magill *et al.*, 1994) and for species between groups. Indeed it has been suggested that there is a continuum of seed desiccation tolerances (Berjak and Pammenter, 1994).

The species examined in this chapter cover a range of desiccation tolerances and putative seed storage categories. Four species were chosen all of which were tropical to contrast with the temperate species already studied;

- *Azadirachta indica* (neem)

  A socio-economically important tropical species whose seeds, are reputedly difficult to store using conventional seed banking methods. From the family *Meliaceae*, neem is a large evergreen tree, 12 to 18 m in height with a straight trunk and long spreading branches. It is native to India and Burma, but the tree has also been introduced to, and is found growing successfully in, Central America, the Caribbean, South America, Asia and Africa. The fruit of the Neem tree is a one seeded drupe, which contains an oval seed about 13 mm in length (Ng, 1991).

- *Araucaria angustifolia* (Brazilian pine)

  The *Araucarias* are coniferous trees of the *Araucariaceae* family; some species are important in forestry for plantation (Nikles, 1980 and Ntima, 1968). Most species derive from regions with a tropical or sub-tropical climate.

- *Theobroma cacao* (cacao)

  Cacao is a strictly tropical crop, restricted in cultivation to lowland areas where the annual rainfall is around 200 cm, this creates the high levels of humidity which are ideal for growth. The genus *Theobroma* is a member of the family *Sterculiaceae*, which includes 50 genera and 700 species of tropical trees and shrubs. There are

about 20 species in the genus *Theobroma*, but only *T. cacao* is of major economic importance.

- *Carica papaya* (papaya)

  Belonging to the family *Caricaceae*, a small group of four genera of trees or shrubs, with leaves in terminal clusters and latex vessels running throughout their tissues. There are about 40 species of *Carica* in the American tropics and sub-tropics, and the paw paw or papaya probably originated in Central America, perhaps as a hybrid between other species. A hermaphrodite cultivar ('solo') is used commercially for food and for it's latex and papain content, papain is used extensively in the food processing industry.

5.2 Experimental design

5.2.1 Desiccation treatments

The origin of the seeds used is described in Table 2.1, they were either commercially purchased (neem - Green Gold International, Ludhiana, India; papaya - Henry Mears of Lewes, East Sussex) or donated (Brazilian pine - Dr A Mederios, EMBRAPA-Cenargen, Brazil; cacao - Dr P Headley, Reading University, UK). Upon arrival at Wakehurst Place they were briefly stored at either 2, 15 or 16 °C (see Table 2.2) before experimentation. They were then either immediately sampled (controls) or desiccated in the dry-room for varying amounts of time, seeds of each species being sampled at appropriate intervals (determined by % eRH measurements, see Chapter 2); up to 840 h for neem, 672 h for Brazilian pine, 40 h for cacao and 192 h for papaya. Moisture contents were then determined gravimetrically, as previously described (Chapter 2).

Five individual seed moisture contents were made per treatment, at a component tissue level; this consisted of the testa and rest of seed for neem, the bract (seed coat), embryo and female gametophyte for Brazilian pine, embryonic axis and cotyledon for cacao and testa (seed coat), endosperm and embryo for papaya. Seeds were always directly re-hydrated on 1 % agar distilled water at 26 °C for germination studies (n = 2 x 25 for neem and papaya seed, and 2 x 20 for Brazilian pine and cacao; papaya seed required a specific germination treatment which is discussed in detail in Chapter 7).

5.2.2 Measurement of conductivity and respiration

Determinations of electrolyte leakage as carried out for neem, Brazilian pine and cacao seeds, were as previously described (section 4.2.3) using 3 replicate seeds per treatment. Conductivity measurements were taken from the embryo(nic) axes (Brazilian pine and cacao), and rest of seed material (neem, Brazilian pine and cacao) every 20 min for 3h. Respiration rates were investigated for the same species and tissues using the Gilson submarine single valve differential respirometer as previously described (section 4.2.4).

5.2.3 Measurements of oxidative stress

Free radical activity within various seed components of all species was measured using the Bruker ESP-300E X-band spectrometer as previously described (Chapter 2), spectra being recorded as first and second derivatives at -196 °C (only first derivative spectra were recorded for Brazilian pine as the signal : noise ratio was low, preventing second derivative scanning). Biochemical investigations were conducted on a whole seed basis (testa removed) for neem; embryo and female gametophyte for Brazilian pine; embryo and cotyledons for cacao. Four biochemical assays were used. The measurement of aldehydic components was conducted using the commercially available Bioxytech LPO-

586 assay kit, which is specific for 4-HNE and MDA, as previously described (Chapter 2). Measurement of lipid peroxidation consisted of two assays, the commercially available LPO K-assay (LPO-CC) kit, and the fluorimetric determination of TBARS measured as MDA equivalents, as previously described (Chapter 2).

5.3 Results

5.3.1 Physiological effects of desiccation

An exponential decrease in % eRH and moisture content was seen for neem seeds (testas removed) upon desiccation (Fig 5.1A), with eRH falling from c. 72 % to c. 30 % after 400 h of drying. Moisture content fell in line with eRH (Fig 5.1B), from c. 14 % to c. 4 % over the same time period. Brazilian pine seeds showed a slower decline in moisture content on desiccation (Fig 5.2). Moisture was lost most rapidly from the bract of the seed, with all but 10 % of the moisture lost within the first 7 d of desiccation, falling from 35 to 10 %. Loss of water from the embryo and the female gametophyte was much slower. Moisture contents fell from 50 to 40 % after 28 d of desiccation. This decline was linear (r^2 = 0.83) throughout the desiccation procedure (Fig 5.2). Cacao embryonic axis moisture content fell from c. 72 to 42 % after 40 h of desiccation (Fig 5.3), whereas the cotyledon moisture content fell from c. 37 to 18 %, within the first 20 h of desiccation, with a slower reduction in moisture thereafter. Loss of water from papaya seeds was very rapid (Fig 5.4), with moisture content declining from c. 68 to 7, 23 to 9 and 19 to 6 % for the seed testa, endosperm and embryo respectively, within a 24 h period.

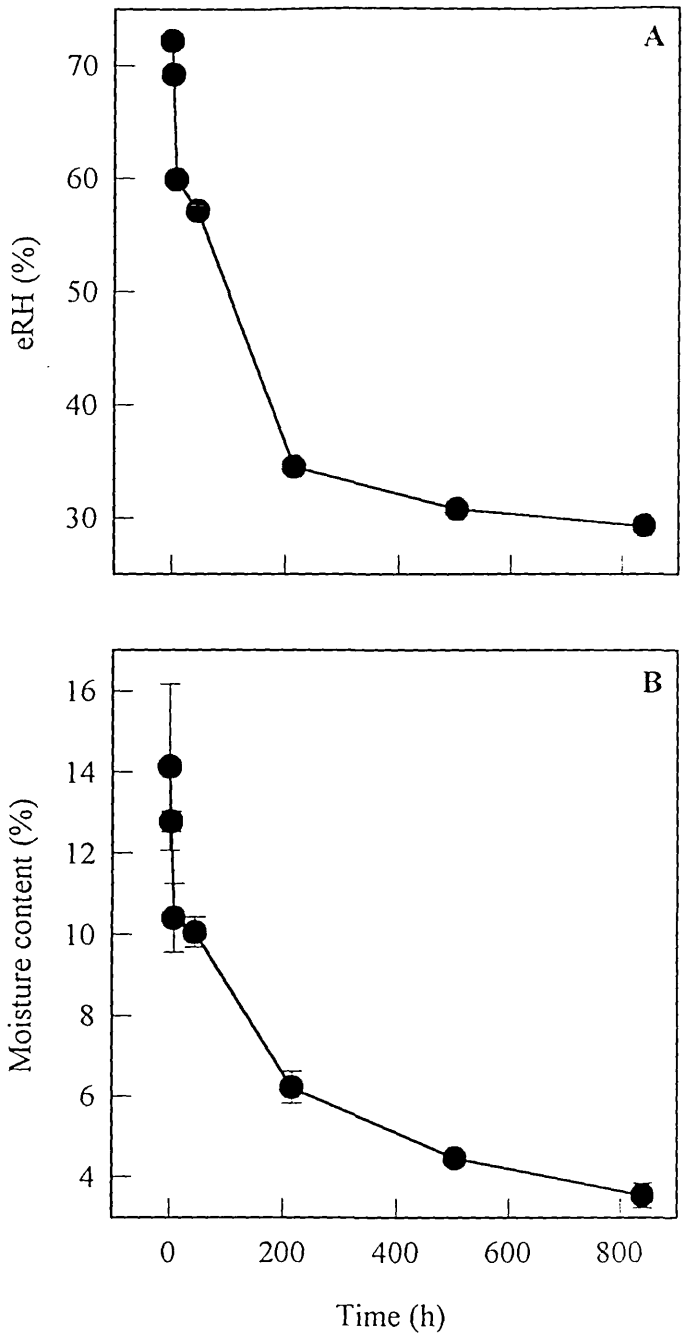


Figure 5.1

Effect of desiccation pericd on the % eRH (A) and moisture content (B) of *Azadirachta indica* A. Juss. seeds (mesotesta removed). Error bars represent one s.d. of the mean.

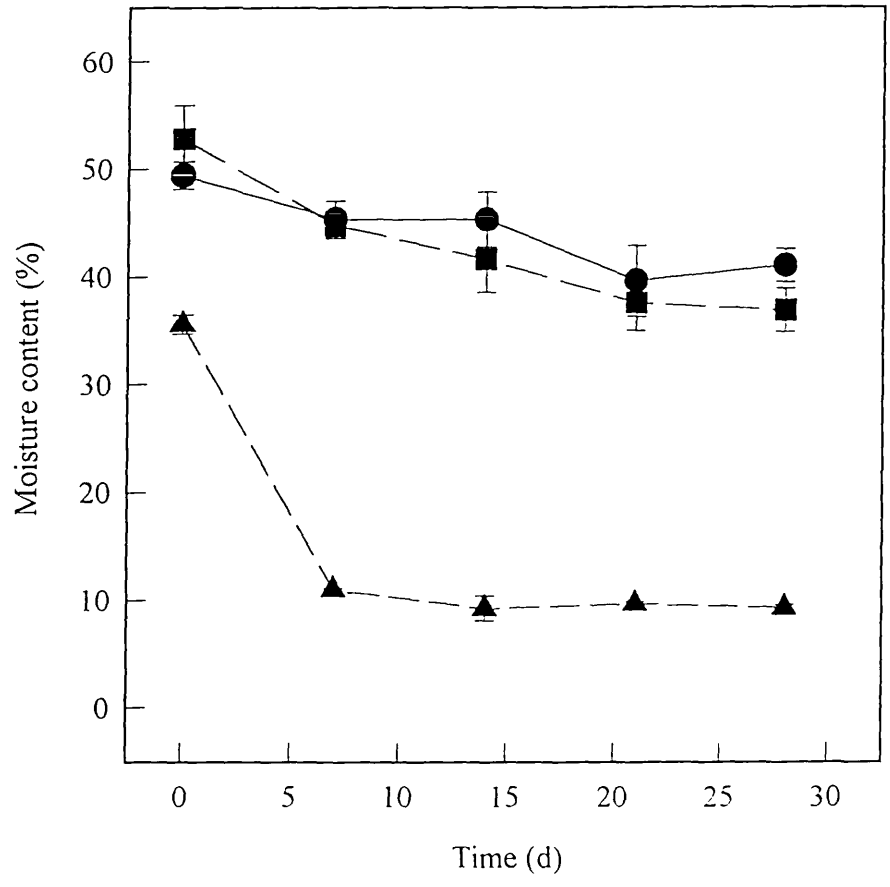


Figure 5.2

Effect of desiccation period on the moisture content of the embryo (circles), female gametophyte (squares) and bract (triangles) of *Araucaria angustifolia* L. seeds. Error bars represent one s.d. of the mean.

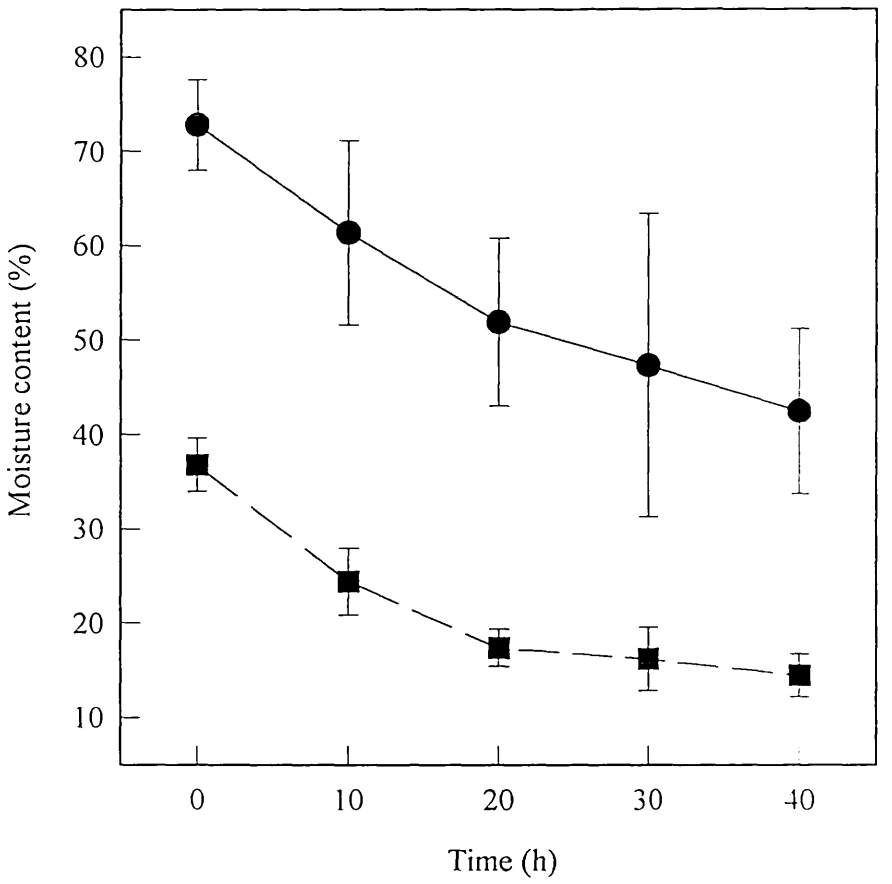


Figure 5.3

Effect of desiccation period on the moisture content of the embryonic axis (circles) and cotyledons (squares) of *Theobroma cacao* L. seeds. Error bars represent one s.d. of the mean.

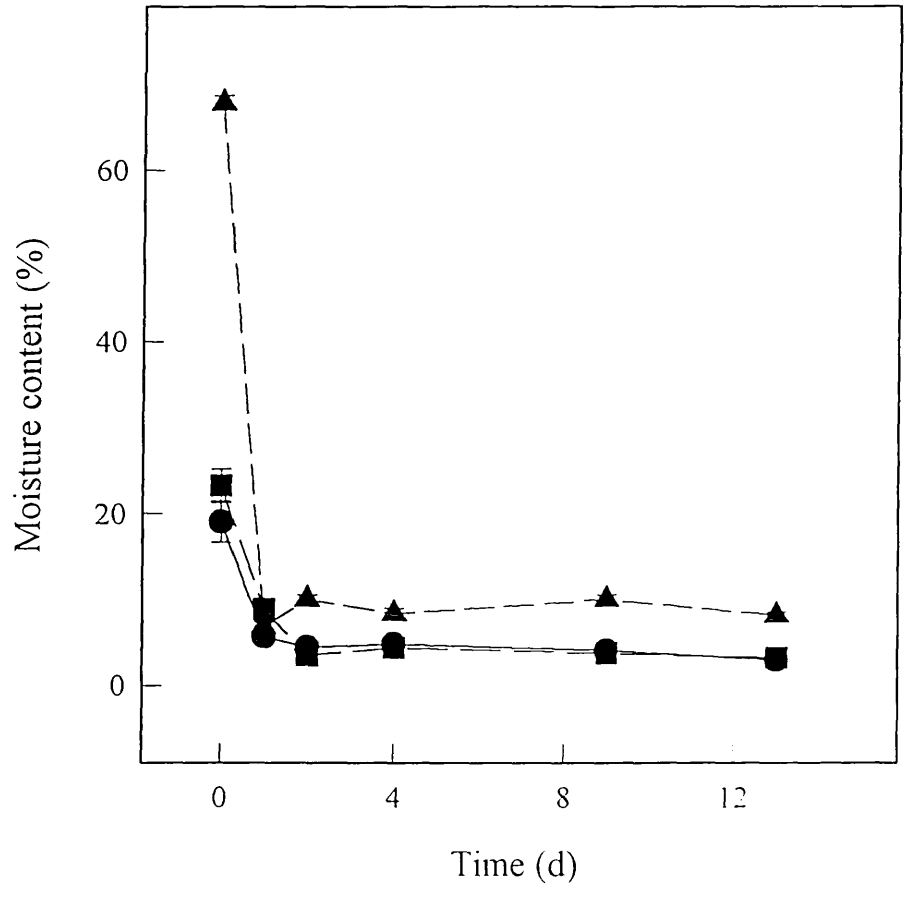


Figure 5.4

Effect of desiccation period on the moisture content of the embryo (circles), endosperm (squares) and testa (triangles) of *Carica papaya* L. seeds. Error bars represent one s.d. of the mean.

Initial germination of neem seeds was relatively high at c. 62 %, however, upon desiccation this increased to a maximum of c. 85 % at 11 % moisture content, before falling to c. 37 % at 4 % moisture content (Fig 5.5A). On an equilibrium moisture content basis (Fig 5.1A), desiccation to c. 55 % eRH was not detrimental to viability. Further drying to 35 % eRH halved viability, but lowering to an eRH of 30 % did not reduce germination further. Brazilian pine seeds also initially displayed a high viability (Fig 5.5B) of about 80 %. However, upon desiccation below 50 % moisture content for the embryo, viability immediately dropped, and continued to decrease to zero as moisture fell to 40 %. The initial viability of cacao seeds was 100 % (Fig 5.5C). However, desiccation from 70 % to 60 % axis moisture content halved viability, a final germination level of c. 30 % was reached at 40 % moisture content. Papaya seeds survived desiccation to very low moisture contents (c. 9 %; Fig 5.5D), once a desiccation-induced dormancy response was overcome (see Chapter 7).

Electrolyte leakage increased upon desiccation in neem seeds (Fig 5.6), from c. 0.3 to 0.7 μmhos after a 180 min soak time. Brazilian pine seeds also displayed an increased electrical conductivity level following desiccation, and rehydration both for the embryo (Fig 5.7A) and female gametophyte (Fig 5.7B). However, in contrast to the female gametophyte the embryonic axis measurements were higher and displayed a clear separation, with electrolyte level increasing dramatically from c. 0.3 to 1.0 μmhos after 30 h of drying (c. 45 % moisture content). Data is unavailable for cacao seeds, as the experiment gave results that were unreliably variable.

Respiratory activity was detected in the embryo of Brazilian Pine seeds (Fig 5.8) where 120 μl of oxygen was consumed after 120 mins incubation. Results for the female

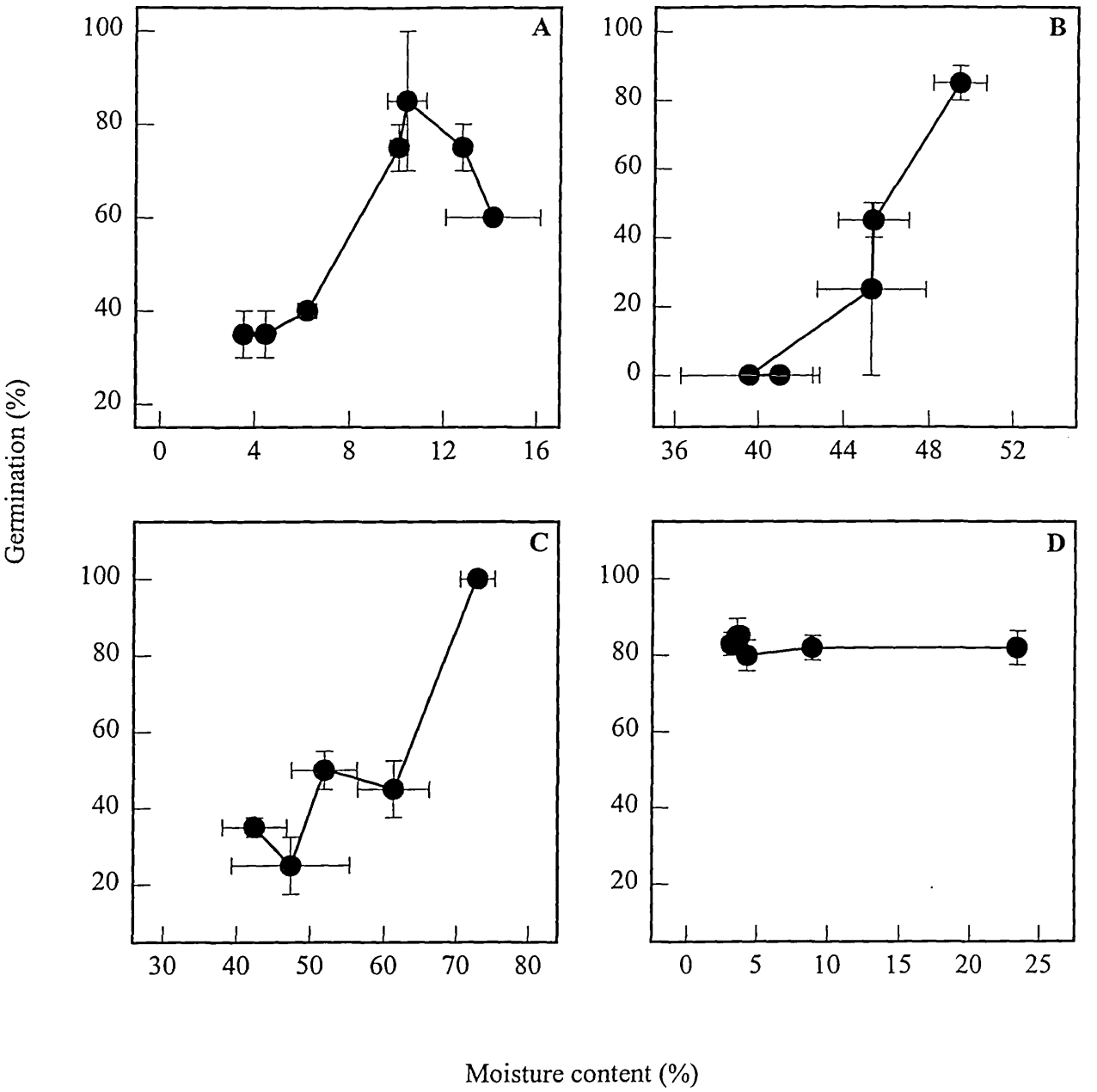


Figure 5.5

Relationship between the moisture contents of (A) *Azadirachta indica* A. Juss. seed (mesotesta removed), (B) *Araucaria angustifolia* L. seed embryo, (C) *Theobroma* cacao L. seed embryonic axis and (D) *Carica papaya* L. seed endosperm and germination. Error bars represent one s.d. of the mean.

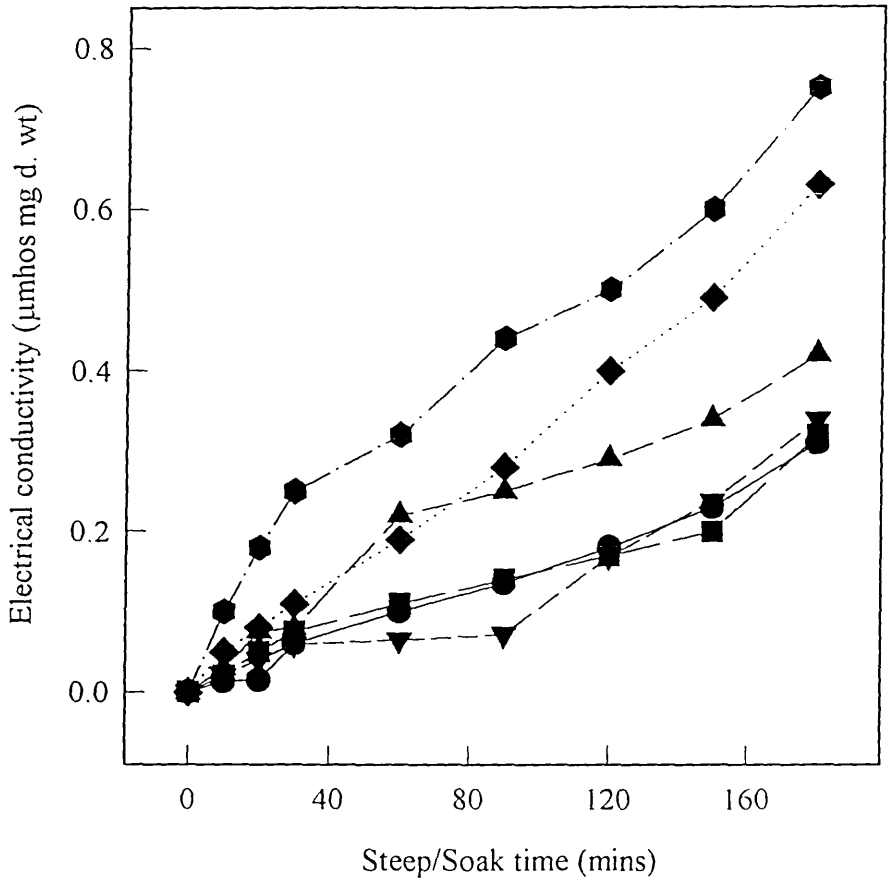


Figure 5.6

Relationship between electrical conductivity and desiccation for three *Azadirachta indica* A. Juss. seeds (mesotesta removed). Symbols represent c. 14 % (circles), 13 % (squares), 10 % (triangles), 6 % (inverted triangles), 5 % (diamonds) and 4 % (hexagons) moisture content (f. wt. basis).

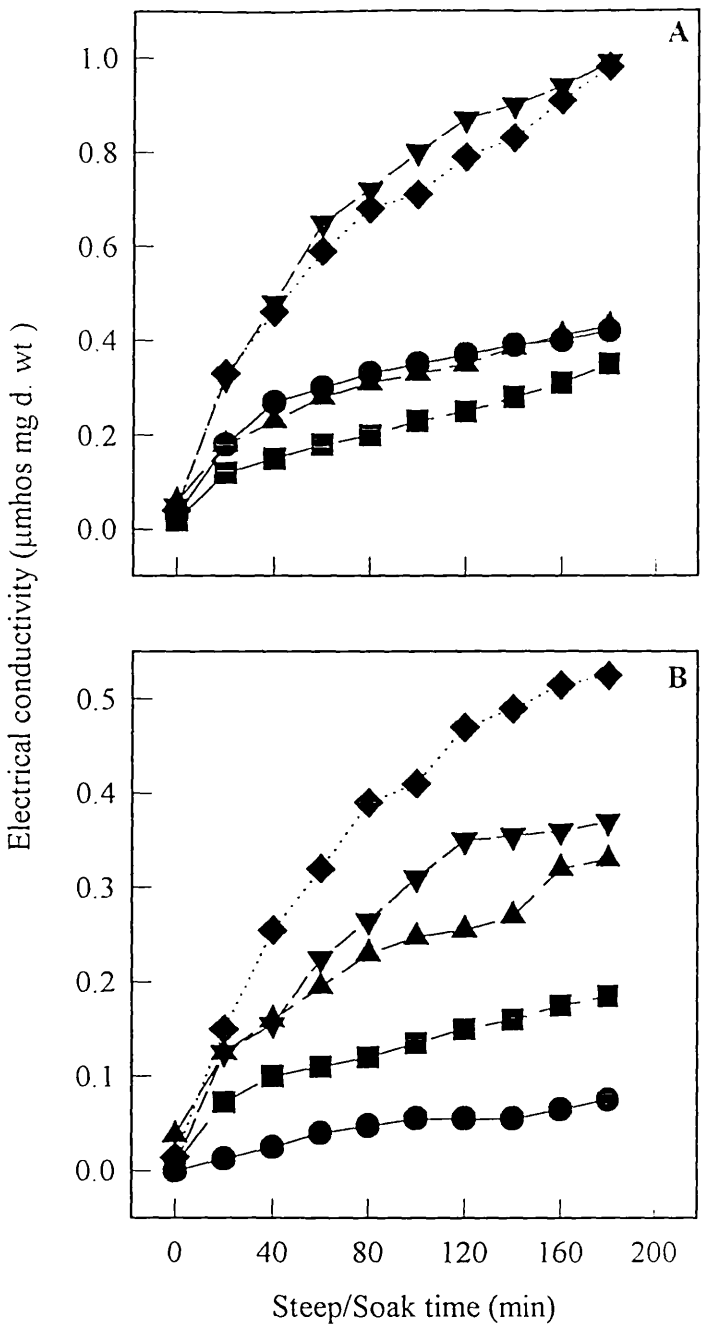


Figure 5.7 Relationship between electrical conductivity and desiccation for three *Araucaria angustifolia* L. seed embryos (A) and female gametophytes (B). Symbols represent c. 49, 53 % (circles), 48, 45 % (squares), 47, 42 % (triangles), 43, 38 % (inverted triangles) and 41, 37 % (diamonds) moisture content (f. wt. basis) for the embryo and female gametophyte respectively.

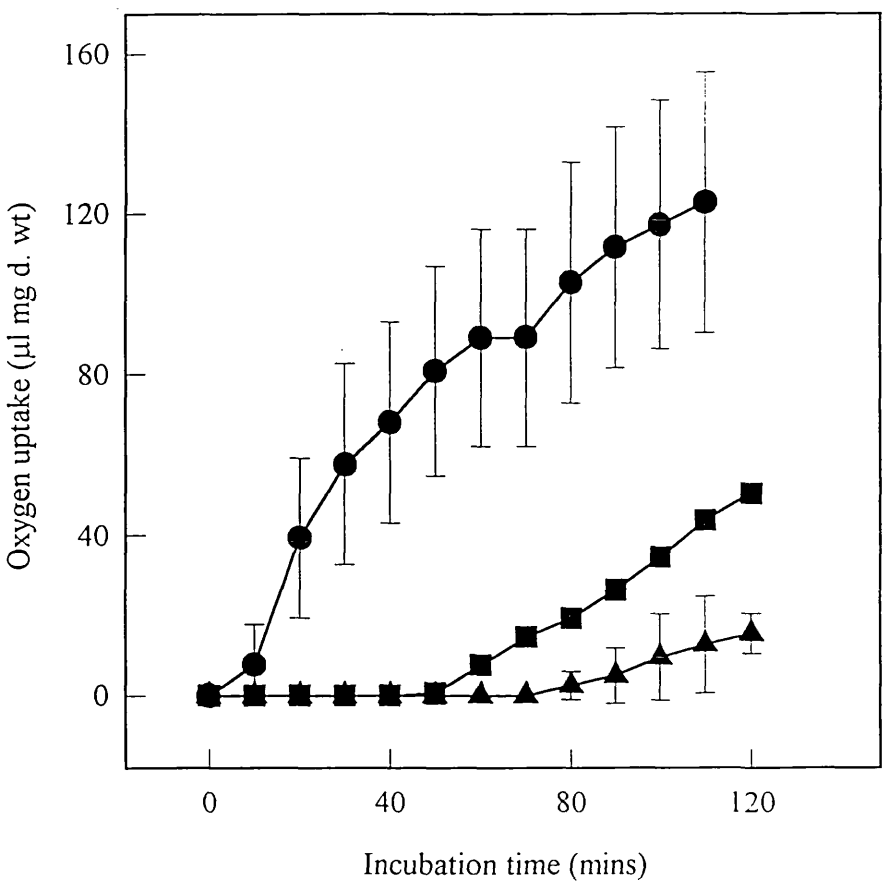


Figure 5.8

Time course of oxygen uptake for three *Araucaria angustifolia* L. seed embryos (circles), female gametophytes (squares) and bracts (triangles). Error bars represent one s.d. of the mean.

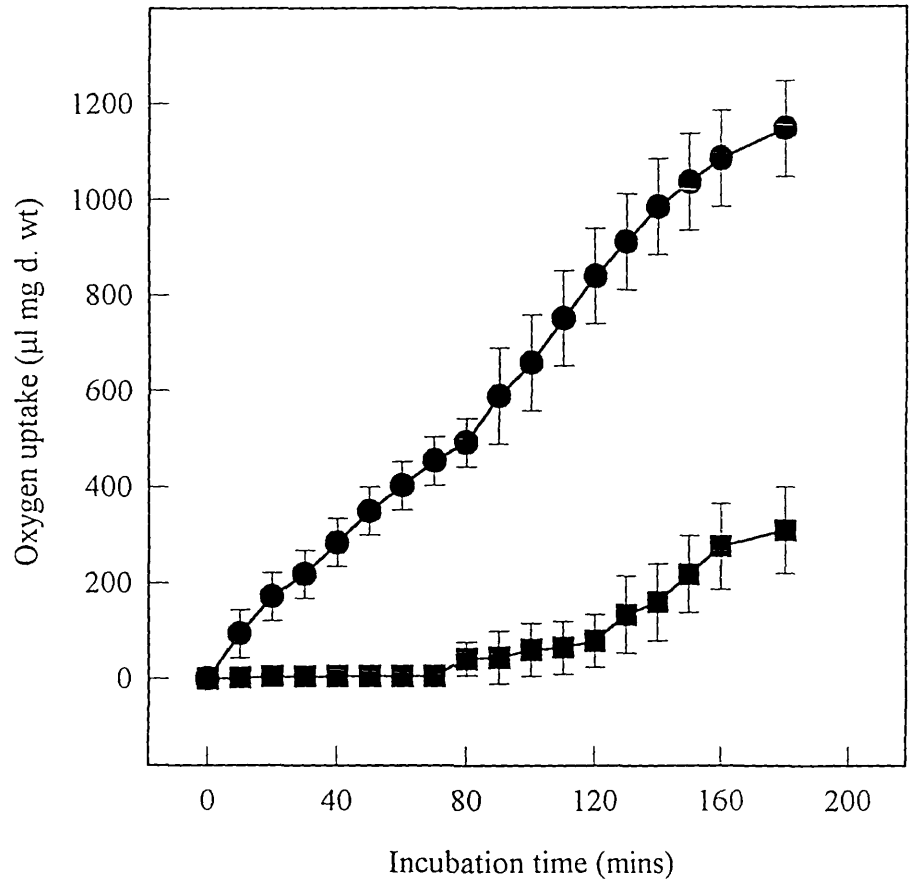


Figure 5.9

Time course of oxygen uptake for three *Theobroma cacao* L. seed axes (circles) and cotyledons (squares). Error bars represent one s.d. of the mean.

gametophyte and seed bracts were much lower, consumption reaching c. 50 and 15µl respectively after 120 mins. The rate of respiration appeared to be bi-phasic for the embryo, increasing with incubation time. However, respiration of the female gametophyte and bract was undetectable until > c. 60 mins incubation, coincidental with the second phase of increasing oxygen uptake in the embryo; thereafter there was a linear ($r^2 = 0.93$) increase. Overall the rate of respiration in this species was low. Cacao embryonic axis gave much higher rates of respiration than did the cotyledons (for control samples see Fig 5.9). Over 1 ml of oxygen was consumed from undried embryonic axes during a 180 min incubation period. Cotyledonary tissue oxygen uptake only reached 300 µl during the same time period. Oxygen uptake of neem seed was undetectable on several repeat runs, as a result of the low initial moisture content of the seed (i.e. c. 14 %).

5.3.2 Biochemical effects of desiccation

In neem seed (mesotestas removed) concentrations of 4-HNE were seen to transiently increase (Fig 5.10A) from c. 2.4 to 3.3 mmol per g d. wt around the point of viability loss (c. 6 to 10 % moisture content). This overall trend was repeated for MDA, however the increase occurred almost immediately upon desiccation, but rising from c. 2 to a peak of 7.5 mmol per g d. wt at around the point of viability loss (Fig 5.10B). Between 4 to 6 % moisture content there was a decrease in MDA concentration. Similar trends were also seen for total lipid peroxidation (LPO) and TBARS (Figs 5.11A and B).

4-HNE was seen to increase from c. 11 to 22 mmol per g d. wt in Brazilian pine seed embryo's, again around the point of viability loss (c. 46 % moisture content) for the embryo (Fig 5.12A). The level of this chemical within the female gametophyte and

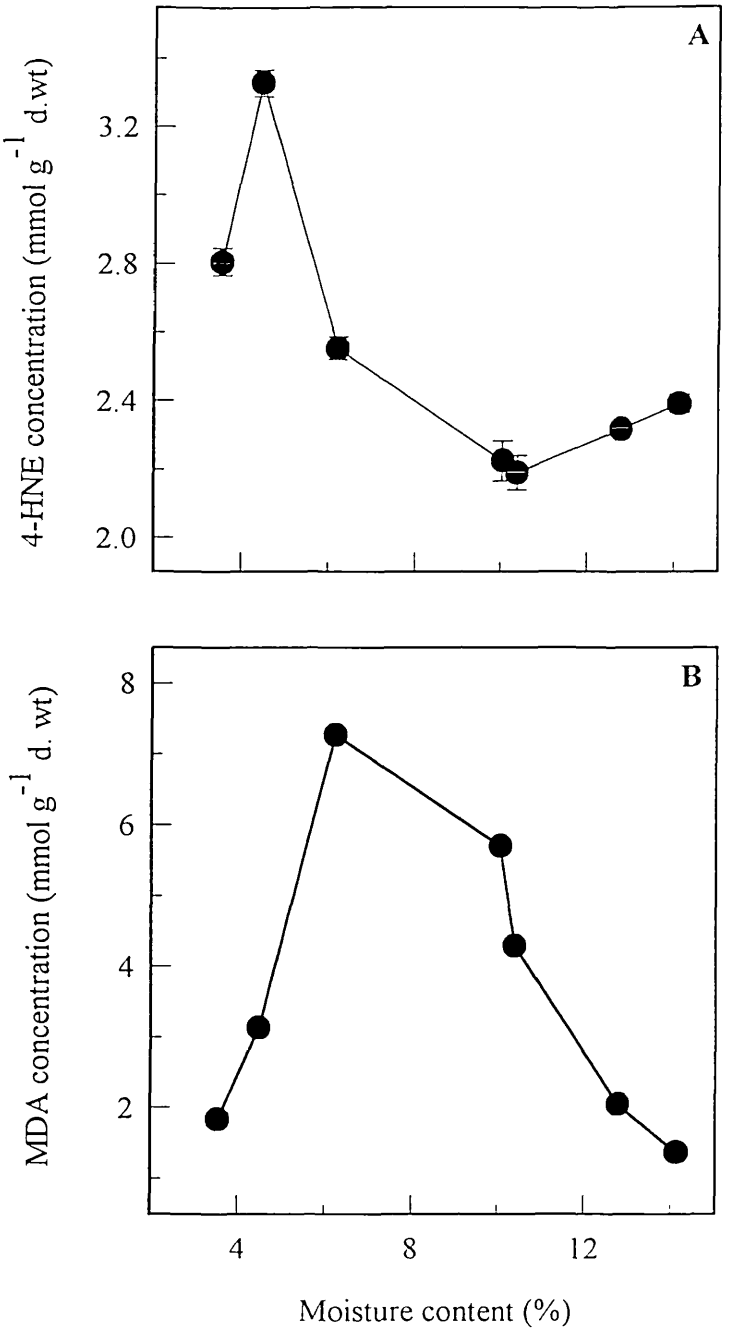


Figure 5.10

Relationship between both the 4-HNE (A) and the MDA concentrations (B) and the moisture content of *Azadirachta indica* A. Juss. seeds (mesotesta removed). Error bars represent one s.d. of the mean.

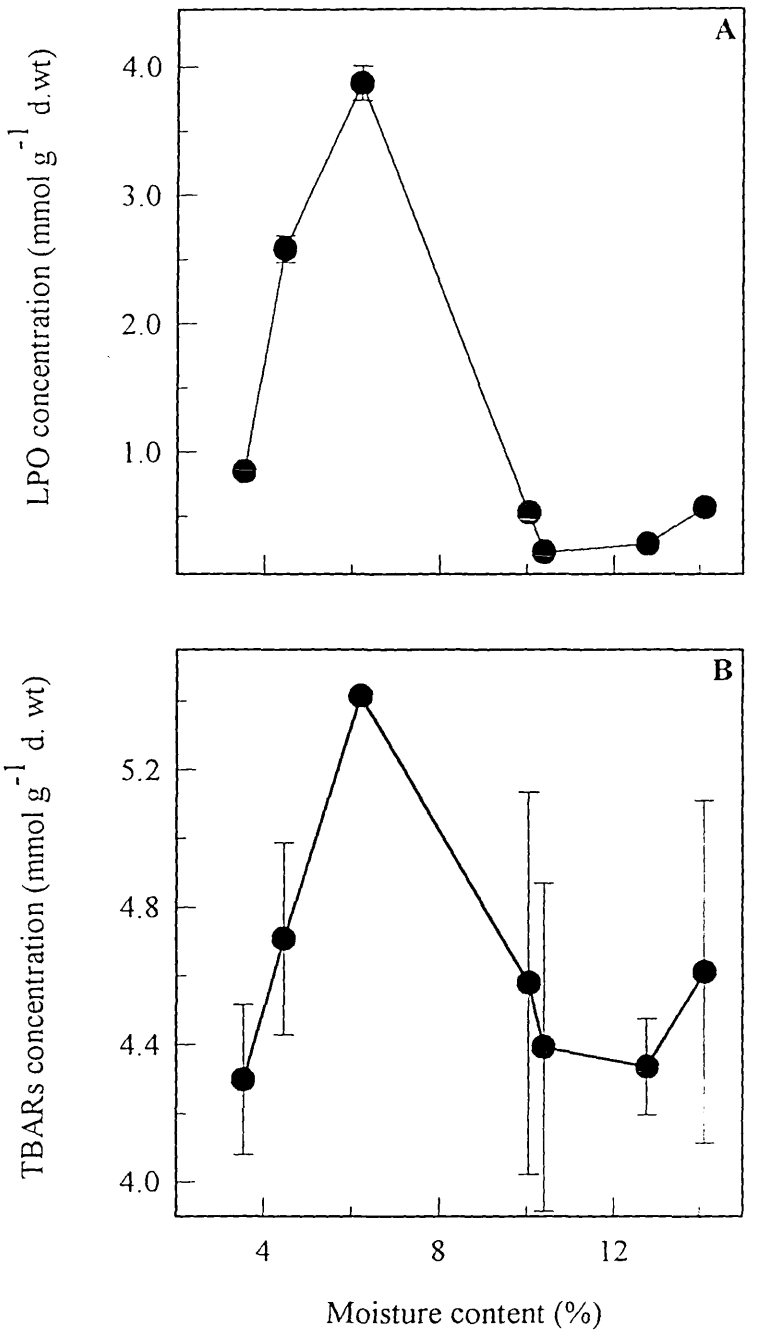


Figure 5.11

Relationship between both the LPO (A) and the TBARS concentrations (B) and the moisture content of *Azadirachta indica* A. Juss. seeds (mesotesta removed). Error bars represent one s.d. of the mean.

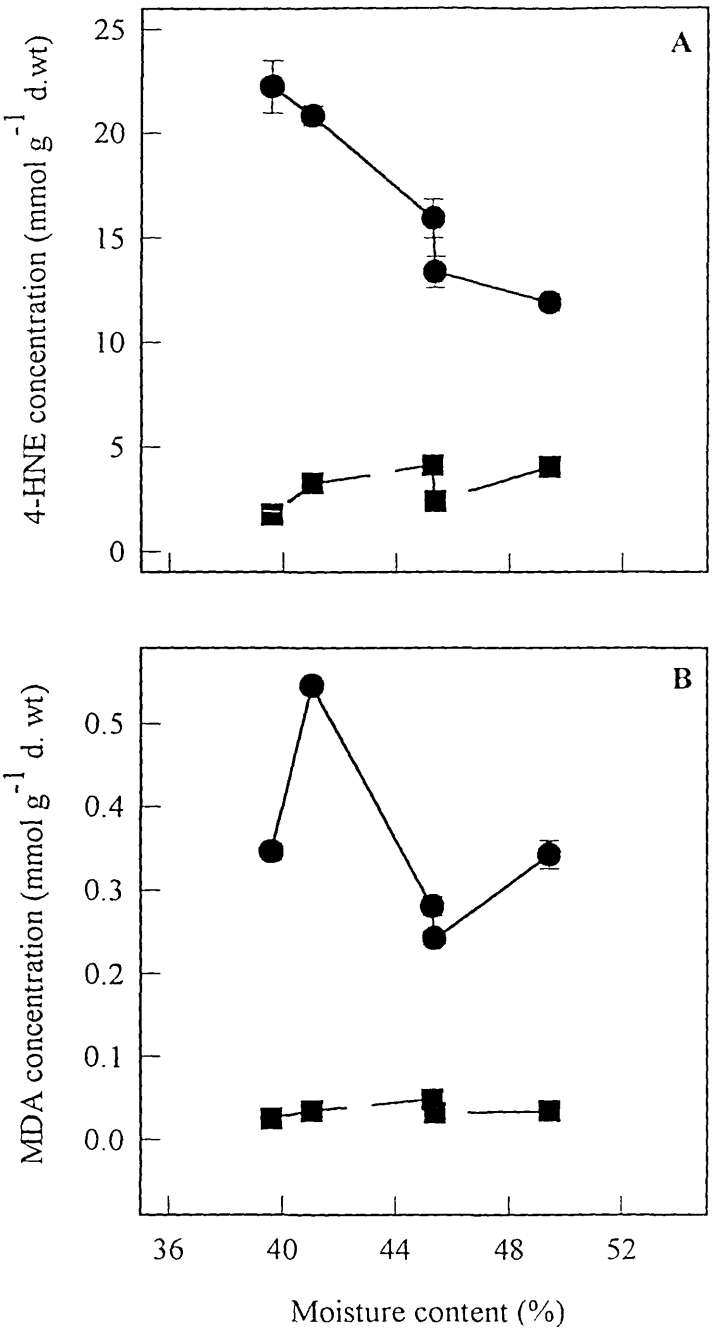


Figure 5.12

Relationship between both the 4-HNE (A) and the MDA concentrations (B) and the moisture content of *Araucaria angustifolia* L. seed embryos (circles) and female gametophytes (squares). Error bars represent one s.d. of the mean.

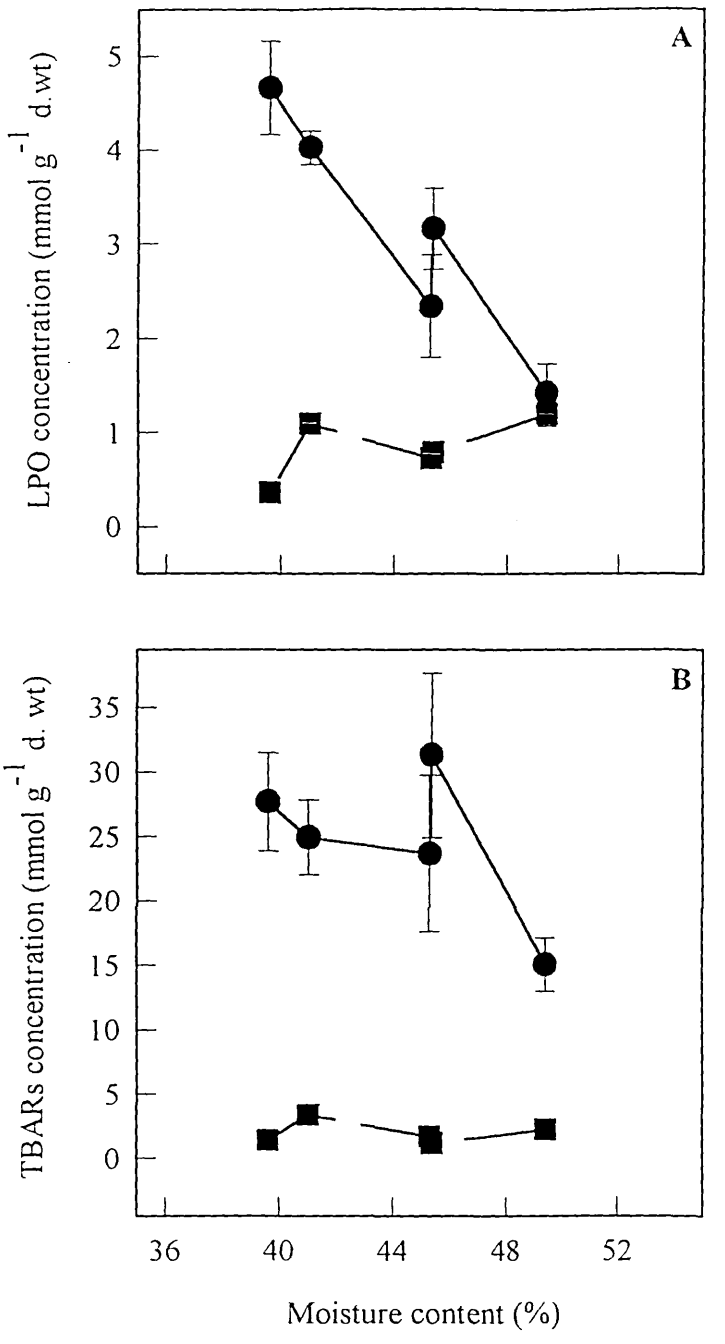


Figure 5.13

Relationship between both the LPO (A) and the TBARS concentrations (B) and the moisture content of *Araucaria angustifolia* L. seed embryos (circles) and female gametophytes (squares). Error bars represent one s.d. of the mean.

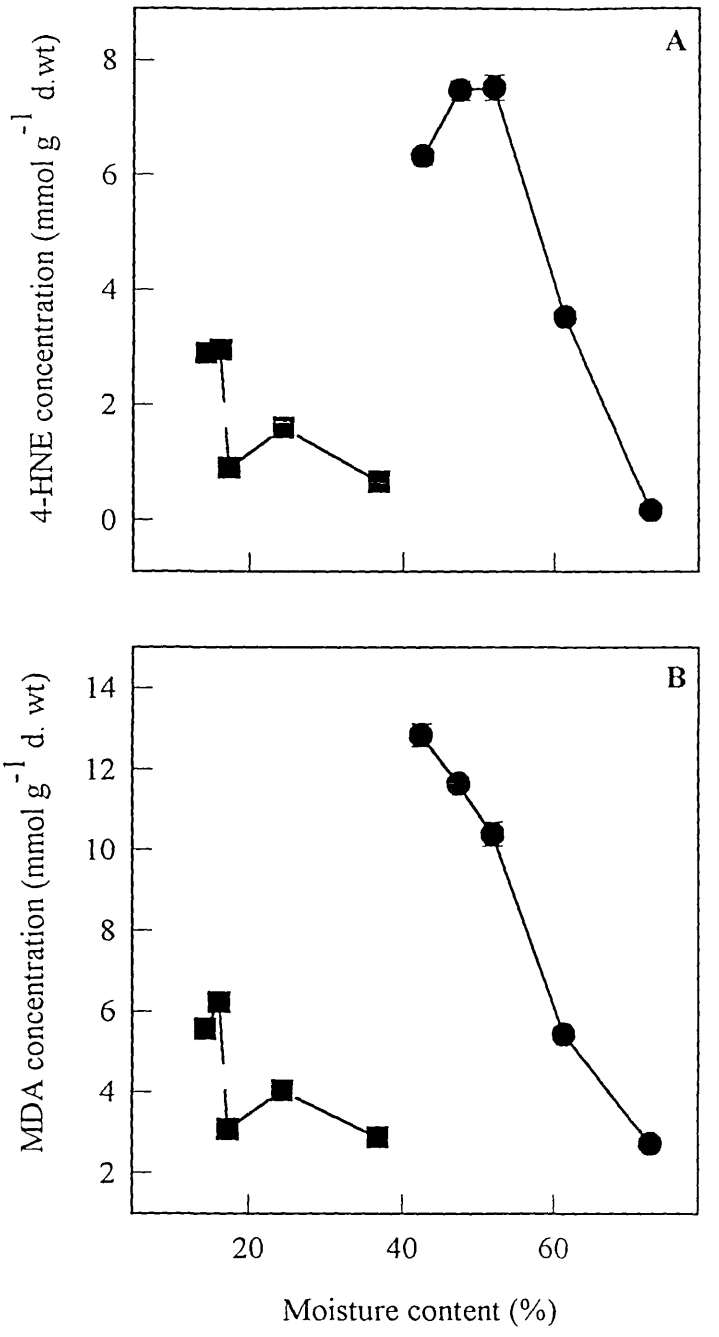


Figure 5.14

Relationship between both the 4-HNE (A) and the MDA concentrations (B) and the moisture content of *Theobroma cacao* L. seed embryonic axes (circles) and cotyledons (squares). Error bars represent one s.d. of the mean.

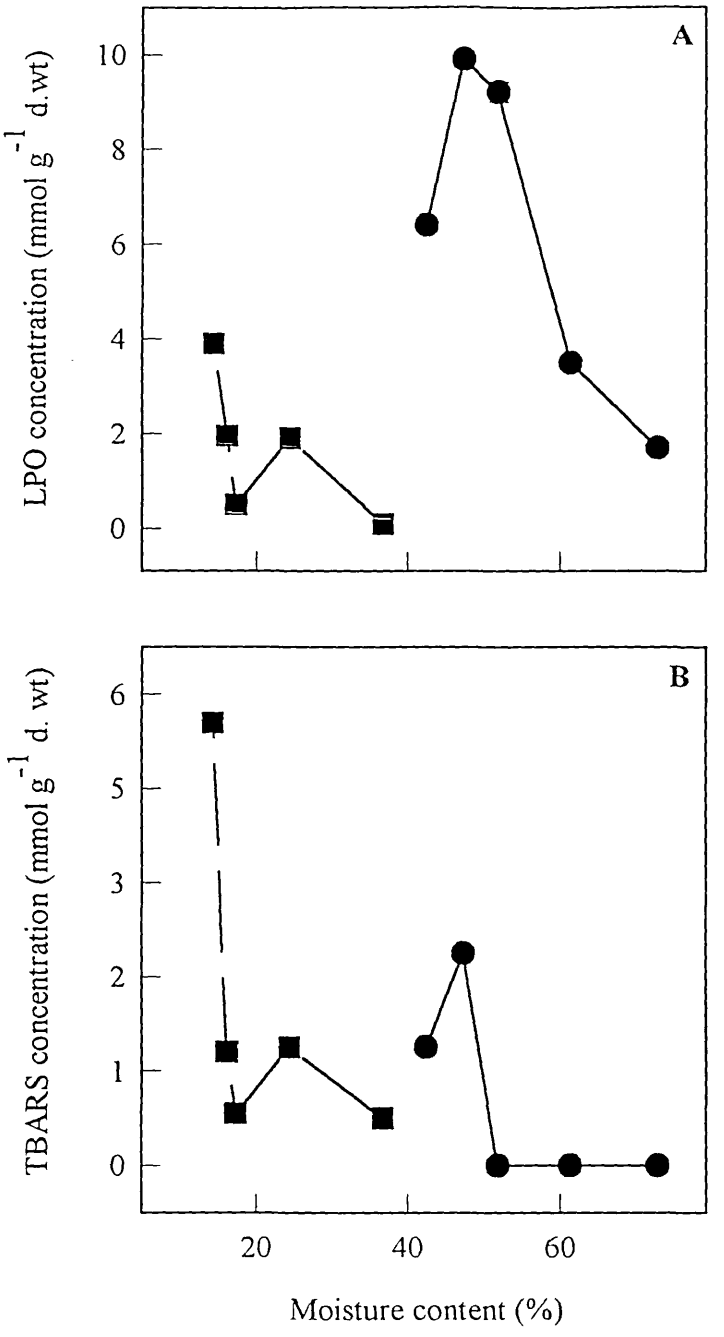


Figure 5.15

Relationship between both the LPO (A) and the TBARS concentrations (B) and the moisture content of *Theobroma cacao* L. seed embryonic axes (circles) and cotyledons (squares). Error bars represent one s.d. of the mean.

bract (data for the bract is omitted from Figs 5.12 and 5.13 for reasons of clarity) remained constant at < 5 mmol per g d. wt. This overall trend was repeated for MDA (Fig 5.12B), rising from c. 0.3 to 0.55 mmol per g d. wt, again at the point of viability loss. Similar trends to that seen for 4-HNE were also displayed for total lipid peroxide and TBARS levels (Figs 5.13A and B).

Increases in lipid peroxidation products were also seen in cacao seed upon desiccation. 4-HNE concentration increased from c. 0.2 to 8.0 mM per g d. wt again coincidental with the point of viability loss (c. 60 % moisture content) within the embryonic axis (Fig 5.14A). Increases in 4-HNE for the cotyledon were much lower, but still very apparent, the increase taking place at c. 15 % moisture content. The same general trends were observed for MDA (Fig 5.14B) and LPO levels (Fig 5.15A) in desiccating tissues of the axis and the cotyledons. Overall, 4-HNE, MDA and LPO increased in the axes and cotyledons by c. 6 and 3-fold respectively. In contrast, the relative increase in TBARS on viability loss was reversed in the cotyledons and the axis at the point of viability loss, reaching c. 6 and 2.5 mmol g^{-1} d. wt. respectively (Fig 5.15B).

5.3.3 Biophysical effects of desiccation

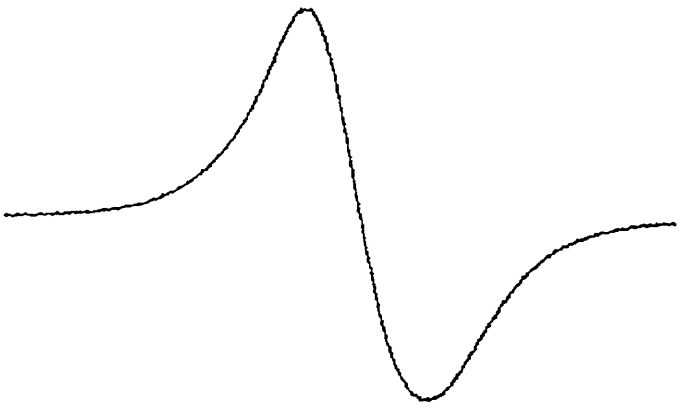
In neem seed there was an overall rise in free radical levels (Table 5.1) upon desiccation. With increasing desiccation there was a tripling of free radical LF signal intensity (Fig 5.16B; g-value 2.0150 ± 0.050) around the point of viability loss (c. 6 % moisture content), the identification of a HF signal was not possible.

Brazilian pine seeds showed low levels of free radical activity, and the resultant low signal : noise ratio prevented second derivative scanning. However, an overall rise in

Table 5.1
EPR determined free radical signal intensities (both LF and HF) for Azadirachta indica, Araucaria angustifolia, Theobroma cacao *and* Carica papaya *seed tissues.*

Species	Moisture content (%)	LF signal intensity mm g^{-1} d. wt. x 10^{-2}	HF signal intensity mm g^{-1} d. wt. x 10^{-2}
Azadirachta indica[1]	14.1 ± 2.0	2.509	-
	10.4 ± 0.8	1.252	-
	6.2 ± 0.4	6.251	-
	3.5 ± 0.3	2.253	-
Araucaria angustifolia[2]	49.4 ± 1.3	1.059	1.596
	45.3 ± 1.6	1.414	0.544
	45.1 ± 2.5	1.911	1.054
	41.0 ± 1.5	4.472	2.683
	39.6 ± 3.3	4.662	2.797
Araucaria angustifolia[3]	62.8 ± 3.1	0.594	0.114
	44.8 ± 1.3	0.205	0.097
	41.6 ± 3.0	0.897	0.580
	37.6 ± 2.6	0.780	0.336
	36.9 ± 2.0	0.205	0.108
Theobroma cacao[4]	72.8 ± 4.8	5.010	0.241
	61.3 ± 9.8	5.153	0.252
	51.8 ± 8.9	6.320	0.632
	47.4 ± 16.1	9.260	0.762
	42.4 ± 8.7	9.912	0.513
Theobroma cacao[5]	36.8 ± 2.8	0.220	0.120
	24.4 ± 3.5	0.624	0.223
	17.4 ± 2.0	0.525	0.219
	16.2 ± 3.4	0.411	0.150
	14.4 ± 2.3	0.721	0.141
Carica papaya[6]	23.4 ± 1.9	3.698	0.823
	8.9 ± 6.9	1.723	0.783

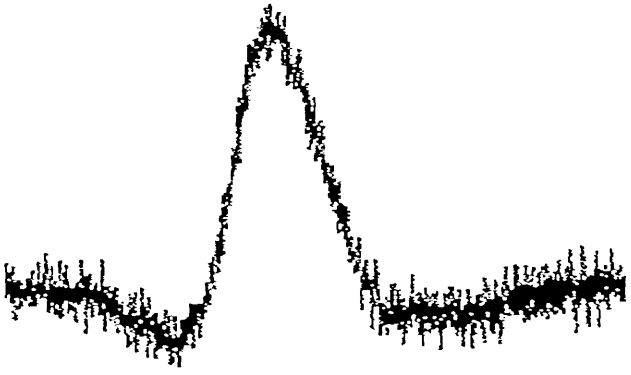
[1] Whole seed, mesotesta removed
[2] Embryo
[3] Female gametophyte
[4] Embryonic axis
[5] Cotyledons
[6] Whole seed, testa removed



10 Gauss

Figure 5.16A

Averaged first derivative EPR spectra from 3 runs, showing the single LF peak signal derived from 'naked' neem (*Azadirachta indica* L.) seeds with mesotesta removed. All measurements were conducted at -196 °C using the rapid-freeze technique (receiver gain = 1.0e + 06). Embryo moisture content was 14.7 ± 0.3 %.



10 Gauss

Figure 5.16B

Averaged second derivative EPR spectra from 3 runs, showing the single LF peak signal derived from 'naked' neem (*Azadirachta indica* L.) seed with mesotesta removed. All measurements were conducted at -196 °C using the rapid-freeze technique (receiver gain = 2.5e + 05). Embryo moisture content was as above.

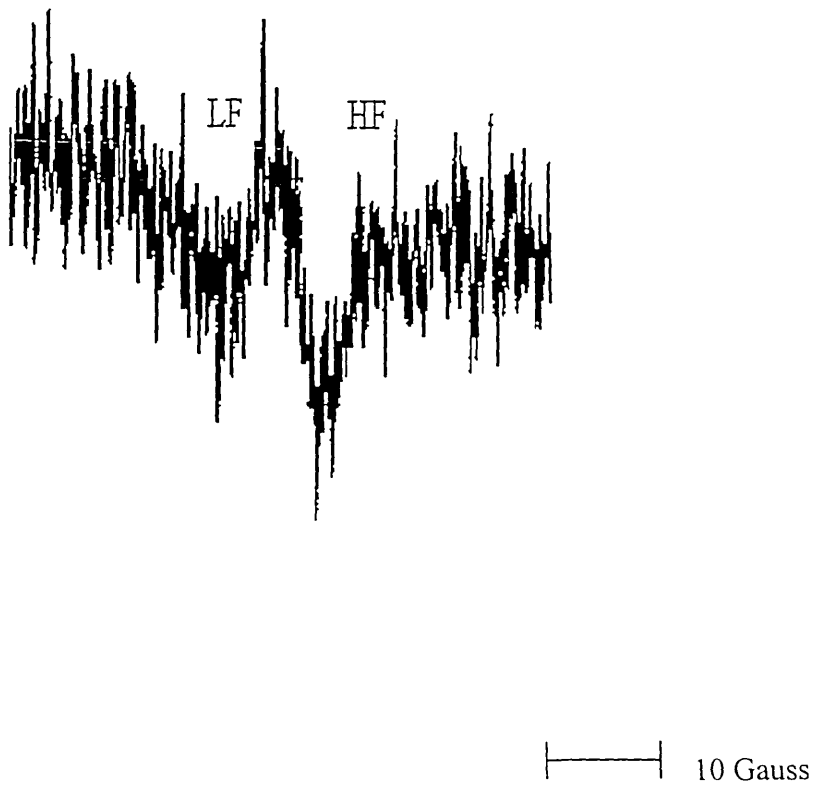


Figure 5.17

Averaged first derivative EPR spectra from 3 runs, *suggesting* a twin peaked signal derived from Brazilian pine seed embryos (*Araucaria angustifolia* L.). Moisture content was 49.8 ± 0.2 %. All measurements were conducted at -196 °C using the rapid-freeze technique (receiver gain = 2.5e + 05).

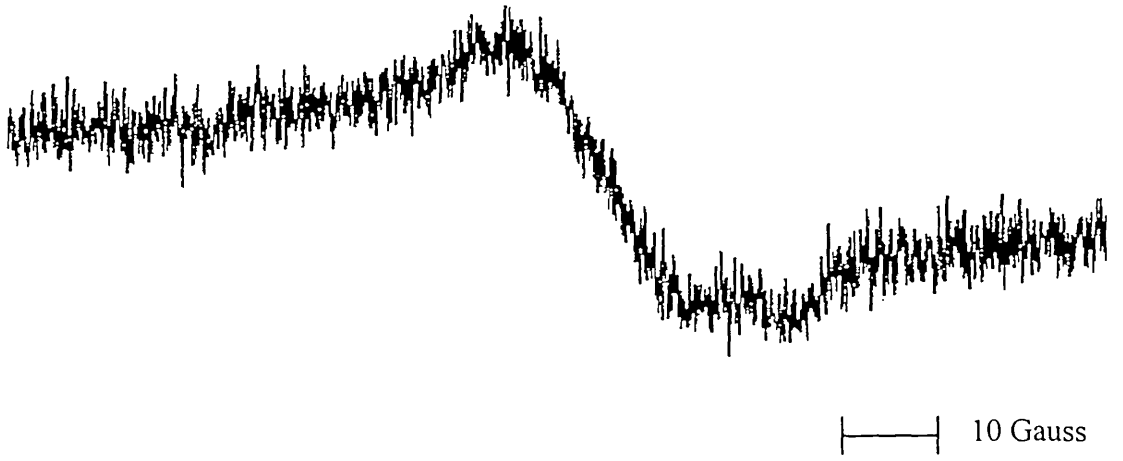


Figure 5.18A

Averaged first derivative EPR spectra from 3 runs, showing the single peak signal derived from cacao (*Theobroma cacao* L.) seed axes. All measurements were conducted at -196 °C using the rapid-freeze technique (receiver gain = 1.0e + 06). Embryo moisture content was 72.3 ± 0.6 %.

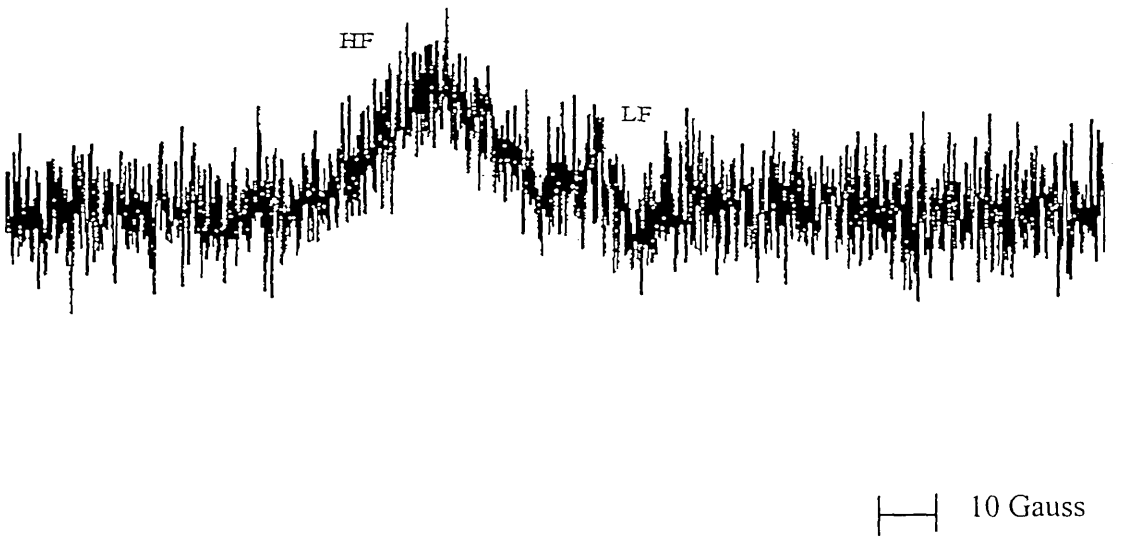


Figure 5.18B

Averaged second derivative EPR spectra from 3 runs indicating two components, showing possible low (LF) and high field (HF) peaks derived derived from cacao (*Theobroma cacao* L.) seed axes. All measurements were conducted at -196 °C using the rapid-freeze technique (receiver gain = 2.5e + 05). Embryo moisture content was as above.

free radical level was observed for both seed components upon desiccation (Table 5.1). With increasing desiccation there was a tripling of the LF free radical signal (Fig 5.17; g-value 2.0016 ± 0.010) around the point of viability loss (c. 42 % moisture content) for the embryo (Table 5.1). The trend was the same to a much lesser degree for the HF signal (g-value 2.0001 ± 0.005), which was less apparent than in the other seed systems studied. This trend was generally repeated in the female gametophyte, however, the increase in free radical signal intensity was more transient for both the LF and HF signals (Table 5.1). A comparison of the LF-signal intensity upon desiccation within the embryo to that of the female gametophyte, showed that the increase in signal intensity occurred at similar (c. 45 - 41 %) moisture contents.

Desiccation of cacao seeds resulted in an increase in free radical levels within both the axis and cotyledons (Table 5.1). Within the embryonic axis a doubling in free radical signal intensity was recorded for the LF, and just over a tripling in signal intensity for the HF (Fig 5.17B; g-values of 2.0113 ± 0.020 and 2.0087 ± 0.010, respectively). The cotyledons displayed a rapid 3-fold increase in the LF signal, and a transient doubling of the HF signal.

Papaya seeds showed an approximate halving in LF signal intensity upon drying (Table 5.1; g-value 2.0041 ± 0.010). The HF signal (Fig 3.9B; g-value 2.0008 ± 0.005) remained constant (Table 5.1).

5.4 Discussion

The purpose of this study was to assess whether the coincidence between increased oxidative activity and viability loss in horse chestnut seed was also a feature of other seeds with differing desiccation tolerances. Results from the previous chapter demonstrated that seeds of a temperate recalcitrant species exhibit increased oxidative reactivity upon desiccation, which was coincidental with the point of viability loss. Other studies have shown similar responses to desiccation using comparable (i.e. temperate) material (Hendry *et al.*, 1990 and Finch-Savage *et al.*, 1994). These observations raise two questions: whether all desiccation sensitive material elicits an oxidative response upon desiccation; and, if so, whether the response can be used as a diagnostic marker for non-orthodox responses to desiccation. For the latter to be the case the response would need to be observable in seed of distinctly different desiccation tolerances.

5.4.1 Physiological responses to desiccation

Measurement of % eRH during the drying of neem seed accurately reflected the rate of moisture loss from the whole seeds, and gave an indication of the most appropriate time intervals to remove seeds from the drying room. There were no evident signs of dormancy for neem, Brazilian pine and cacao, all germinating easily at 26 °C. Papaya seeds, however, proved to be deeply dormant, and required a short term temperature alteration from 26 to 36 °C in order to germinate (this subject, and detail of the response to desiccation are discussed in Chapter 7). High initial rates of germination for neem, Brazilian pine and cacao at 26 °C support the hypothesis that seeds of tropical origin require high thermal optima to germinate (Côme and Corbineau, 1992).

The different rates of viability reduction upon drying, between species, confirms that they display differential sensitivity to desiccation. Viability loss upon desiccation, occurred in seed species at moisture contents which reflected this differential sensitivity. Thus, critical moisture contents ranged from c. 52 to 45 and 8 % for cacao to Brazilian pine to neem.

5.4.1.1 *The response of neem seed to desiccation*

Although the seeds are reportedly short lived (Roederer and Bellefontaine, 1991, Ezumah, 1986, Venkatesh *et al.*, 1990 and Maithani *et al.*, 1989), initial germination of neem seed was readily obtained by using a temperatures of 26 °C, as found by Ezumah (1986) who also had success at 30 °C. Germination was also greatly enhanced by removing the cartilaginous endocarp, as suggested by Chaney and Knudson (1988). Previous work conducted on neem seed desiccation has suggested that it is not as recalcitrant as was previously thought (Lauridsen and Souvannavong, 1993). It may be intermediate or even a short lived orthodox seed (Chaney and Knudson, 1988), as some seeds have tolerated desiccation down to 5 % moisture content without a significant effects on viability (Nagaveni *et al.*, 1987). There is however, a wide variation in responses to drying between different varieties of neem.

Neem of Asian origin appears to be recalcitrant, while African neem has been described as both recalcitrant (Berjak *et al.*, 1995) and orthodox (Gamene *et al.*, 1994 and Tompsett, 1994). A batch of seeds studied by Gamene *et al.*, 1994 from Burkina Faso demonstrated an intermediate behaviour, with partial desiccation tolerance. Using this evidence Berjak *et al.*, (1995) characterised neem as a tropical recalcitrant after

Bonner's classification. Tompsett (1994) studied seeds taken from the Kew Seed Bank which exhibited up to 80 % germination after 12 years of hermetic storage at -20 °C (4 % moisture content). In contrast only a few seeds from lots collected in Barbados and Africa have survived desiccation to 4 % moisture content, and none survived 6 - 8 months at -20 °C (Hong *et al.,* 1996). Suffice to say the characterisation of neem desiccation sensitivity is far from resolved, and in this study the precise storage classification of neem is problematic as there was a variable response within the population to drying.

The slight increase seen in the germination of neem seed upon initial drying (Fig 5.5A) may be a result of maturation (as seen in batch 2 seeds of horse chestnut; Chapter 4). However, the result is more probably due to sampling error associated with the small number of seeds used per germination test, rather than any one particular physiological event. Viability was reduced, however, by 6 % moisture content, although some seeds still survived. This response may be related to differential maturity of the seed lot. Physiologically mature neem seeds (indicated by maximum germination capacity) are usually obtained 10 - 12 weeks after flowering, when some of the fruits turn yellow and start to fall (Maithani *et al.*, 1989). The optimum collection time for these fruits is usually short (c. 1 - 3 weeks). However, I had no control over when, or how, the collection was made and the results suggest a seedlot of heterogeneous developmental ages. As the desiccation tolerance of seed populations progressively improves on maturation drying (e.g. Norway maple; Hong and Ellis, 1992), a percentage of the seed population may therefore, have become tolerant to desiccation injury. Supporting evidence come from Sacande (1995) who has demonstrated that desiccation tolerance in neem can affect only some seeds within a population, this finding was related to

maturation levels. From my studies it would appear that half of the neem seed population exhibited desiccation intolerance, the remainder displaying more typically orthodox behaviour, as one may expect from a collection of seeds displaying a heterogeneous mix of developmental ages.

5.4.1.2 *The response of Brazilian pine seed to desiccation*

The critical moisture content at which Brazilian pine embryos lost viability coincided closely with that reported for this species by Salmen-Espindola *et al.*, (1994). However, the finding that the embryo of this species exhibits a lower desiccation tolerance than the storage tissues (female gametophyte) or whole seed is in contrast to the findings of Salmen-Espindola *et al.*, (1994). This may be related to the fact that drying rates were slightly faster in my study (because of the use of isolated tissues), and this can affect the relationship between moisture content and viability loss (King and Roberts, 1979; Roberts *et al.*, 1984, Vertucci and Farrant, 1995 and Fu *et al.*, 1993).

The embryos of Brazilian pine seeds were seen to be highly metabolically active, as revealed by oxygen uptake measurements. It also appeared that a loss of germinability through desiccation was associated with membrane damage, shown by increased conductivity measurements. This may be related to extensive lipid peroxidation events occurring in desiccating material. Conductivity measurements from Brazilian pine embryos displayed a clear separation, with a substantial increase occurring after 30 h of drying (c. 41 % moisture content; Fig 5.7A). This is tightly correlated to the point at which viability was lost in this species, a finding which has also been reported by Salmen-Espindola *et al.*, (1994). Irreversible solute leakage has previously been correlated with a loss in viability of desiccation sensitive seeds in response to

dehydration (McKersie and Stinson, 1980; Beckwar *et al.*, 1982; Berjak *et al.*, 1989, Fu *et al.*, 1990). Although similar findings have also been found for desiccation tolerant seeds submitted to various stresses (Hendricks and Taylorson, 1976; Givelberg *et al.*, 1984; Priestley, 1986). Dehydration of Brazilian pine embryos also resulted in a decrease in metabolic activity, respiration being markedly affected (Fig 5.8).

5.4.1.3 *The response of cacao seeds to desiccation*

The recalcitrant behaviour of cacao seeds (Fig 5.5C) confirms the findings of Rhül *et al.*, (1988a) who have shown that cacao embryos cannot survive drying below a moisture content of between c. 17 - 25 %, viability starting to decline from c. 70 % onwards. Cacao seeds seem to be typical of other species producing recalcitrant seeds, as they have a high moisture content (c. 32 - 55 %, f. wt. basis) at the time of shedding (Jänicke 1973; Chin *et al.*, 1984 and Rhül *et al.*, 1988b) supporting the view that recalcitrant seeds may be shed at a physiologically immature state (see section 1.1.1.2).

Rhül *et al.*, (1988a and b) have also demonstrated that changes can occur in sub-cellular structures upon desiccation of cacao seeds. It appears that these sub-cellular structural changes in cell nuclei, dictyosomes, amyloplasts, and lipid bodies are a function of the cells dehydration. It seems that changes in internal structures occur as gradual transitions over a wide range of moisture contents, and it has been suggested that the fusion of lipid bodies (although this could be an artifactual result method used, as SEM studies have previously revealed fusion of lipid bodies upon rehydration; Leprince *et* al., 1998) and the dissolution of their envelope surfaces take place suddenly, and are closely correlated with a loss in viability. Thus, the resulting loss of surface stability may be linked with the loss of germinability.

5.4.2 Biochemical / biophysical responses to desiccation

Increases were seen in the levels of lipid peroxidation products for all metabolically active tissue in three species (generally embryonic tissue) upon desiccation, and this points to an oxidative component to desiccation-induced viability loss in tropical desiccation sensitive material. This indication of increased lipid peroxidation is supported by the increase in solute leakage reported above for two of the species. The increased levels of 4-HNE, MDA, LPO's and TBARS upon desiccation indicates that the reactivity of these chemicals is not isolated to seeds of a more temperate origin. Embryonic material of neem seeds was too small to be used for quantitative biochemical analysis, and hence determinations were made on mainly endospermic tissue. Despite this, the greatest levels of biochemical activity were generally observed within embryonic material (the endosperm of course containing the seed embryo). This may be a reflection of the higher desiccation sensitivity of such material (as seen in horse chestnut seeds, see Chapters 3 and 4), and hence its more suitable use for general diagnostic purposes.

A corollary of the above, and the increases observed in free radical signal intensity (Table 5.1) suggests that similar oxidative responses occur in desiccation sensitive tropical material to that observed in recalcitrant temperate seeds (see Chapters 3 and 4). Displayed as a collapse of cell membrane integrity, possibly through the extensive peroxidation of lipid components within the membrane ultrastructure.

Thus, a sequence of irreversible metabolic events seem to be associated with the dehydration of desiccation sensitive seed material. As reported for horse chestnut seeds, the point of damage initiation appears to be concomitant with viability loss, however, as

before (Chapter 4), it is difficult to know whether it is a cause or consequence of the loss of viability.

Conversely, the ability to survive drying and the subsequent decrease in LF free radical signal intensity (Table 5.1) in papaya seeds indicates a high level of desiccation tolerance, and supports the findings of Magill *et al.*, (1994). Papaya has previously been classified as intermediate in it's seed storage behaviour (Ellis *et al.*, 1990), however, the removal of dormancy in this species (see Chapter 7) leads me to believe that this is not the case. Further evidence provided in Chapter 7, points to the fact that papaya seeds exhibit dormancy upon desiccation and thus the perception of the seeds actual storage characteristics could be masked by this feature. A full physiological investigation of this species response to desiccation, and chilling, are described in detail in Chapter 7.

In considering the desiccation tolerance of tropical seeds further, it is clear from this study that within a typical population of neem seeds there are desiccation tolerant and intolerant individuals. This observation and the results obtained from the desiccation of papaya seed indicate that increases in free radical activity, lipid peroxidation and conductivity upon drying of neem seed may only be related to those seeds within the population which were desiccation sensitive, and lost viability.

In conclusion, all desiccation sensitive seed tissues studied display similar trends of oxidative responses to drying. However, the critical moisture content at which this occurs is species dependent, reflecting the differential desiccation sensitivities of the material examined. These critical moisture contents appear to relate generally to the removal of type III water (see Chapter 3), however, in neem seed the response continues

into the type II water sorption zone, well after the point at which type III water has been removed. This is in contrast to other species studied, where oxidative responses have only been observed upon the removal of type III water. This may indicate that the free radical precursor to the biochemical events observed upon drying of desiccation may differ, depending upon hydration status, and the point at which moisture content becomes critical, and this is species variable.

This hypothesis is further supported by the finding that g-values are dissimilar for the free radical species observed between different seeds, indicating the role of several free radical precursors. In many cases more than one free radical is involved in the response to desiccation, within a species (i.e. displaying both a LF and HF signal). Moreover, I have also shown in the population of neem seeds which display desiccation sensitivity that the second derivative free radical signal has a different line shape to that seen in other desiccation sensitive material, only a LF signal being observed. This is in contrast to previous theories which suggest a ubiquitous origin for the free radical (precursor) (Atherton *et al.*, 1993).

Whatever the free radical precursor the overall physiological and biochemical response remains the same for all species. This suggests that different, moisture dependent, metabolic (radical) events take place within desiccation sensitive species, to generate similar biochemical, and hence physiological responses to drying.

These findings, in conjunction with the observation that desiccation tolerant papaya seeds display a lowering of free radical activity upon drying strongly suggests that oxidative stress is a factorial component of desiccation sensitivity. It may be that the

determination of free radical events by EPR, in conjunction with biochemical assessments of lipid peroxidation may serve as a useful diagnostic tool in predicting seed survival of desiccation (for example the fluorametric determination of TBARS is the least sensitive assay of lipid peroxidation, which is apparent by the larger error values. However, it is also the cheapest assay to conduct and this may be useful if such techniques are to be applied as diagnostic tools for, say, developing nations). This would enable the rapid identification of species suitable for conservation through seed banking.

In the following chapter, the desiccation and dry storage of papaya (containing mainly type I and II water), will be related to oxidative events occurring during viability loss. For comparison the oxidative response of a recalcitrant seed (containing type I to V water) to hydrated storage and subsequent drying is also reported.

5.5 Summary

Studies on seeds of four species of differing desiccation tolerance revealed that in three species viability loss due to drying was concomitant with changes in oxidative metabolism, both in terms of increasing lipid peroxidation and free radical activity.

The production of these chemicals upon desiccation-induced viability loss, can occur when water removal covers both type II and III water sorption zones. This suggests that there are possibly different biophysical contributions to the viability loss of desiccation

sensitive material, that are related to specific critical moisture contents which are species dependent.

Papaya seed survived desiccation, and there was an associated decrease in free radical activity. This suggests that desiccation-tolerant seeds may have more efficient biochemical protection from oxidative stress, enabling them to survive extreme drying.

CHAPTER 6

A Comparison of the Oxidative Stress Response to Storage of Both Desiccation-intolerant and -tolerant Seeds.

Chapter 6: A comparison of the oxidative stress response to storage of both desiccation-intolerant and -tolerant seeds.

Outlay of Chapter

Chapter 6. A Comparison of the Oxidative Stress Response to Storage of Both Desiccation-intolerant and -tolerant Seeds.

6.1 Introduction

The previous three chapters support the premise that desiccation-induced damage in recalcitrant and putative intermediate seed is associated with a free radical mediated mechanism of injury. In this chapter to extend the study beyond the effects of drying *per se*, an attempt is made to address a further hypothesis; that the expression of desiccation-induced oxidative damage is affected by metabolic events that take place within the seed during storage.

Oxidative stress plays a central role in plant cell viability loss under fully hydrated conditions. In this respect, correlations have been observed between lipid peroxidation and (i) the progressive loss of morphogenic potential of suspension cultures during long term culture (Benson *et al.*, 1992) and (ii) the slow cumulative loss of sprouting potential in stored potato tubers (Kumar and Knowles, 1993). Moreover, oxidative stress has also been shown implicated in the loss of viability in dry-stored orthodox seeds (Reuzeau and Cavalie, 1995, Corbineau and Côme, 1995, Smith and Berjak, 1995 and Bailly *et al.*, 1996). However, the nature of oxidative metabolism in wet and dry material can vary (St. Angelo, 1992), and hence it is quite possible that the mechanisms of viability loss may also differ.

Horse chestnut seeds are desiccation intolerant (Chapter 4; Tompsett and Pritchard, 1993) but are also reported to be relatively long-lived when stored under hydrated conditions which are not conducive to germination (Pritchard *et al.*, 1996). Papaya seeds are putative intermediates, but as seen in the last chapter, can survive desiccation, see also Magill *et al.*, (1994) and Chapter 7. Such seeds, therefore, provide an ideal system to investigate the role of oxidative stress in viability loss due to natural ageing (through hydrated storage) and drying using desiccation sensitive horse chestnut seeds, and dry storage of desiccation tolerant papaya seeds. In this chapter I have characterised the free radical and lipid peroxidation product profiles of seeds during viability loss, related to both desiccation and storage. As such I aim to improve the understanding of the mechanism of seed viability loss at both high and low moisture contents, and facilitate improvements in storage protocols for recalcitrant and non-recalcitrant seed material.

6.2 Experimental design

6.2.1 Seed storage, desiccation and germination

Two species were selected to represent typical recalcitrant and non-recalcitrant seeds, *viz* horse chestnut and papaya, respectively. Batches 1 and 2 (see Chapter 4) of horse chestnut seeds were held in black polythene bags at 16 °C for approximately 1 month after collection (Table 2.2) The seeds were then transferred to agar water in Perspex sandwich boxes (17 x 11 x 7 cm) for hydrated storage; each box containing 15 seeds. The boxes were stored in the dark at the same temperature for up to a further 5 months (batch 1) and 17 months (batch 2). Boxes were inspected approximately every month and infected seeds noted and removed.

For germination, two replicate boxes of seeds per treatment were transferred to 35 °C in the dark, and germination was assessed as radicle emergence to at least 1 cm (Fig 4.6). Seeds were desiccated as a mono-layer in slatted trays placed in the dry-room (as previously described; Chapter 2). Seed rehydration was performed directly on agar water as part of the germination test. Moisture contents of seeds in both the desiccation and hydrated storage experiments were determined individually on seven replicates of embryonic axes and remaining seed tissues (testa and cotyledons combined) as previously described (Chapter 2). Results are presented on a f. wt basis.

Papaya seeds at 15 % eRH were rehydrated over a 3 week period at 21 °C, above a saturated salt solution of $Zn(NO_3)_2.6H_20$ to c. 45 % RH. After removal from the rehydration cabinet, the % eRH was measured as previously described (Chapter 2). For treatment seeds were divided into samples, each of about 80 seeds, and hermetically sealed in glass vials (11 mm diameter, 25 mm long) with a close fitting rubber septa and aluminium crimp (Pierce and Warriner Ltd., Chester, U.K.), and placed in a 40 ± 1 °C incubator in the dark for storage. Each vial was then flushed with either air, oxygen, nitrogen or carbon dioxide to form varying degrees of oxygen concentrations within each vial. The appropriate gas was introduced from a pressurised storage cylinder through a pressure reduction valve via a hypodermic needle. A second needle provided an outlet and was connected to a gas-flow meter. The vial was flushed every 2 d for 1 min at a flow rate of about 45 ml min^{-1}. Measurements showed that this did not effect seed moisture content (data not shown), the method was adapted after that of Ibrahim and Roberts, 1983.

After varying storage periods of up to 72 d, 2 x 25 seeds from each gaseous environment were removed from storage and placed to germinate at 26 °C. To remove dormancy, seeds

were exposed to 36 °C for 4 h, after 14 d at 26 °C (the justification for using this method is described in detail in Chapter 7).

Electrolyte leakage and respiration rate determinations were made on stored and desiccated horse chestnut seeds, samples of both axes and cotyledonary segments (c. 3 x 3 x 3 mm) were measured using batch 2 horse chestnut seed as previously described (Chapter 2). Five replicates of each tissue were used per treatment. Rates of respiration (oxygen uptake) were measured at 35 ± 1 °C using the Gilson submarine single valve differential respirometer as previously described (Chapter 2). Measurements were performed on three individual embryonic axes per treatment and recorded every 10 mins for 2 h. The system was allowed to openly-vent for 10 min before each series of measurements to allow for changes in gas pressure. The low moisture contents of papaya seeds, after equilibration, negated the measurement of respiration and conductivity.

6.2.2 Biochemical determinations

Three assays were used to quantitatively determine the concentration of lipid peroxidation products in horse chestnut and papaya seed components. MDA and 4-HNE were measured using the LPO-586 assay. Total lipid peroxide concentration was measured using the LPO-K (LPO-CC) assay. The concentration of TBARS was also determined by the fluorimetry as previously described (Chapter 2).

The total protein content of horse chestnut seed components was measured using, the Bradford method (Coomassie assay), and antioxidant activity was monitored by assaying for SOD, catalase and peroxidase activity, as previously described (Chapter 2). All enzyme assays were performed at 25 °C and expressed on the basis of total soluble

protein content in relation to standard curves. No antioxidant activity determinations were made on papaya seed.

6.2.3 Biophysical determinations

Free radical determination was performed on excised tissues (axes and cotyledonary segments) from stored and desiccated seeds of both horse chestnut seedlots, and 'naked' (testa / mesocarp removed) papaya seeds, which were rapidly-frozen in liquid nitrogen immediately upon sampling. They were then directly transferred to a Suprasil (pure quartz) finger dewar containing liquid nitrogen, and placed in the EPR cavity. Free radical signal intensity was measured at -196 °C. Other EPR measurement parameters are outlined in Chapter 2.

6.3 Results

6.3.1 Effects of hydrated storage on horse chestnut seed viability

Freshly harvested horse chestnut seeds had embryonic axis and cotyledonary moisture contents of 65 ± 1 % and 51 ± 1 % respectively for batch 1 seed and 69 ± 1 % and 52 ± 1% respectively for batch 2 seed. Storage on agar at 16 °C resulted in a steady increase in seed moisture content by c. 0.6 % per month, reaching 62 ± 4 % over 18 months for the batch 2 material. By comparison, the embryonic axis moisture content only increased slightly to 70 ± 2 % over the same period.

Seed germination characteristics were found to have altered during storage in two ways. Firstly, final germination either increased slightly (batch 2) or remained unaltered (batch 1) after storage periods of up to 12 months, and thereafter decreased by 18 months (Fig

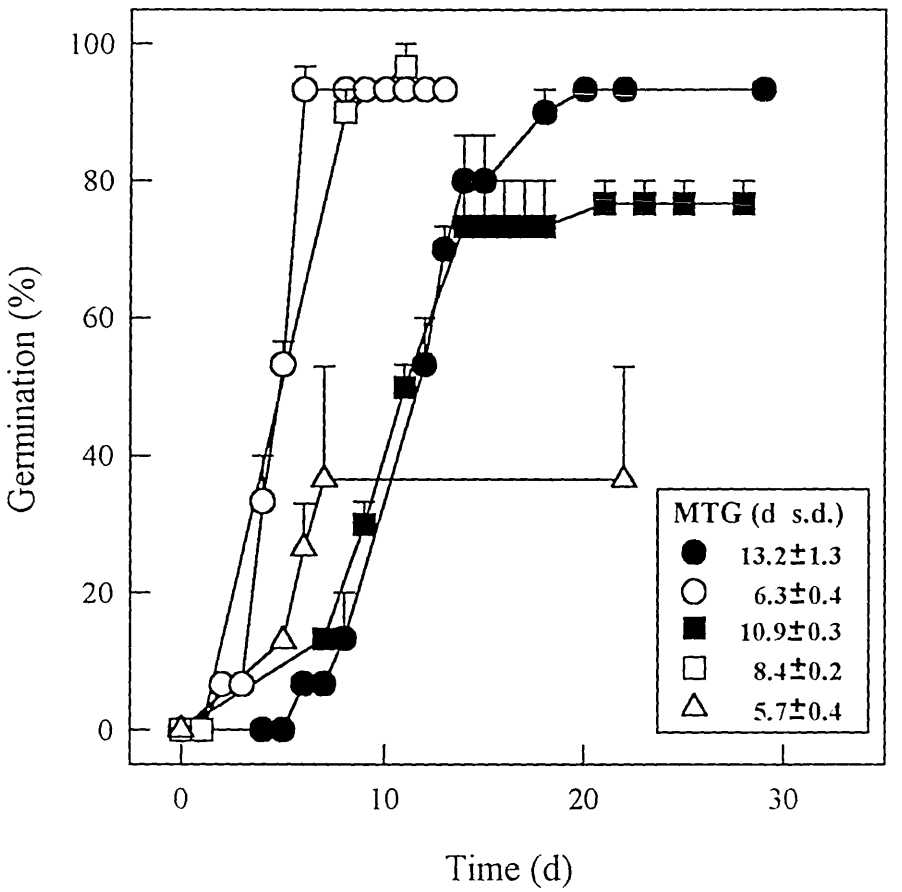


Figure 6.1 Time course for germination of *A. hippocastanum* seed from 1993 (circles) and 1994 (squares, triangles). Seeds were stored hydrated at 16 °C for 0 (●, ■), 6 (○), 12 (□) or 18 months (△). Bars represent one s.d. of the mean. Mean time to germinate (MTG) values are also shown.

Table 6.1. *Effects of drying on the electrical conductivity and respiratory activity of axes extracted from* A. hippocastanum *(batch 2) seeds stored hydrated at 16°C.*

Conductivity and respiration values were recorded after 3 h on three individual axes which had been rehydrated post-desiccation prior to measurement. For the hydrated storage experiment, data are significantly different ($P < 0.05$) when followed by different letters. For desiccation experiments on stored material, data are significantly different ($P < 0.05$) when followed by different symbols.

Pre-desiccation storage time (months)	Axis moisture content after drying (%)	Electrical conductivity (μmhos mg d. wt.)	O_2 uptake (μl mg d. wt.)
0	69 ± 3	9.2 ± 1.2 a *	117.6 ± 4.6 a *
0	36 ± 4	41.0 ± 0.8 +	126.7 ± 5.7 *
0	23 ± 6	46.0 ± 0.8 #	17.2 ± 1.8 +
12	69 ± 2	14.9 ± 1.4 b *	55.8 ± 3.2 b *
12	25 ± 1	58.0 ± 1.2 +	82.3 ± 2.8 +
12	14 ± 1	62.0 ± 0.6 #	20.2 ± 3.0 #
18	70 ± 1	15.6 ± 0.8 b *	96.6 ± 4.5 c *
18	44 ± 18	61.2 ± 1.4 +	98.3 ± 4.6 *
18	24 ± 3	77.2 ± 1.2 #	19.6 ± 3.8 +

6.1). Secondly, compared to the controls the mean time to germinate at 35 °C decreased by 5 - 7 d for all periods of storage from 6 to 18 months (Fig 6.1). However, storage did not result in pre-sprouting of seeds or an accumulation of axis dry weight, e.g. both the control and 6 month stored seeds from batch 1 seed had axis dry weights of 26 ± 8 mg. Axes conductivity was not a reliable indicator of seed germinability *per se* as small increases in leakage rate had already occurred by 12 months hydrated storage (Table 6.1) when germination remained high (Fig 6.1). However, increases in conductivity may have been an indication of the first signs of viability loss in the seed population.

As previously seen (Chapter 4) the cotyledons displayed much lower levels of EPR-determined free radical accumulation and enzymatic protectants of activated oxygen, compared to the axes. For example, cotyledon tissue catalase, peroxidase and SOD activities were 50, 11 and 26 % lower than in the axes (data not shown). As a consequence the remainder of the work presented in this chapter on horse chestnut deals with oxidative stress parameters of the axes.

Axes of highly germinable seed prior to medium-term hydrated storage exhibited EPR-spectra with two distinct peaks, indicative of the presence of free radicals (Fig 3.4). The main peak occurred in the low-field (LF) region and had a g-value of 2.0060± 0.0015; the smaller peak, in the high-field (HF) region, had a g-value of 2.0020 ± 0.0005 (see Chapter 3). Hydrated storage (at c. 69 % axis moisture content) resulted in a transient increase in the axis of both the LF and HF free radical signal intensity, which peaked after about 9 - 10 months and decreased again by 18 months (Fig 6.2). Over the same storage period, axis respiration rates decreased by between 18 and 53 % (Table 6.1).

A range of lipid peroxidation products (TBARS, MDA, 4-HNE and LPO) were present at low levels in seed axes prior to medium-term storage (Figs 6.3 and 6.4). Although after 12 months hydrated storage elevated levels of TBARS, 4-HNE and LPO were observed in the axes of batch 2 seed, lipid peroxidation levels were lower again by 18 months (compare symbols at extreme right within Figs 6.3 and 6.4).

During storage over 18 months the protein content of the axes increased nearly seven-fold to 7 mM mg^{-1} f. wt. (Table 6.2), whereas the content of the cotyledons decreased slightly from 5.0 to 3.7 mM mg^{-1} f. wt. In addition, enzyme activity was undetectable in

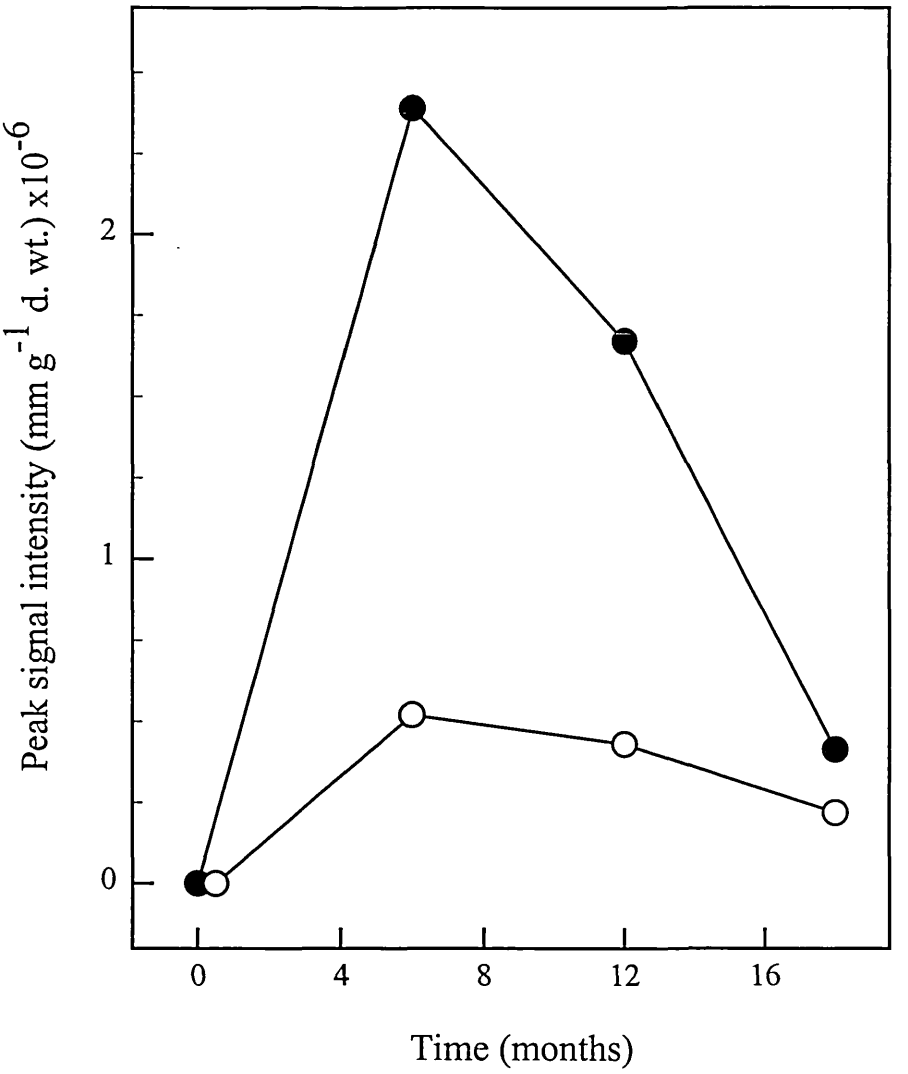


Figure 6.2 Effects of hydrated storage at 16 °C on the LF (closed symbols) and HF (open symbols) EPR signal intensity in axes of *A. hippocastanum*. Combined data from two experiments reported in Fig 6.6 was independently normalised to constant dry weight and zeroed at the start of the storage period. Fitted lines are 1st order polynomials; parameters for the LF and HF responses are $y = 0.066 + 0.476x - 0.026x^2$ ($r^2 = 0.93$) and $y = 0.013 + 0.103x - 0.005x^2$ ($r^2 = 0.95$) respectively.

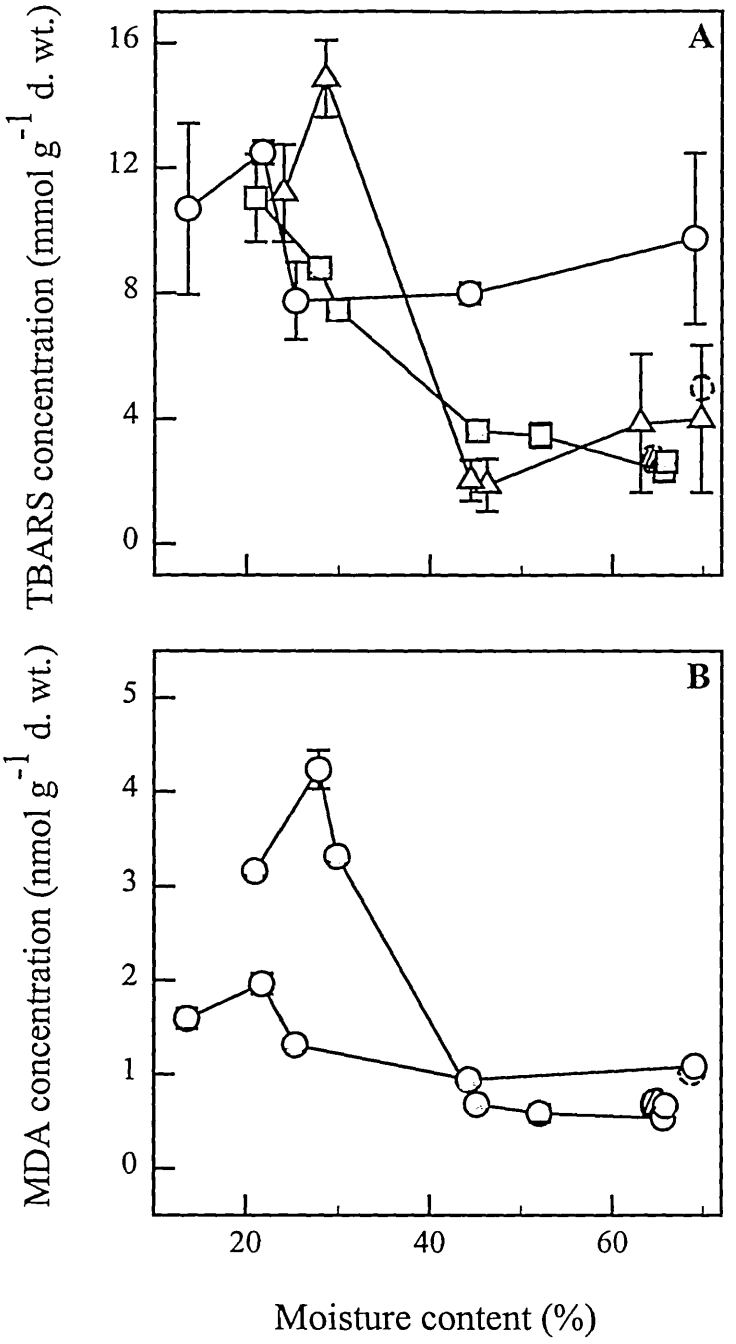


Figure 6.3

Relationship between both the TBARs (A) and the MDA concentrations (B) and the moisture content of *A. hippocastanum* seed axes. Symbols are as in Fig 6.1 and bars represent s.d. Ghosted symbols represent unstored material (see Chapter 4).

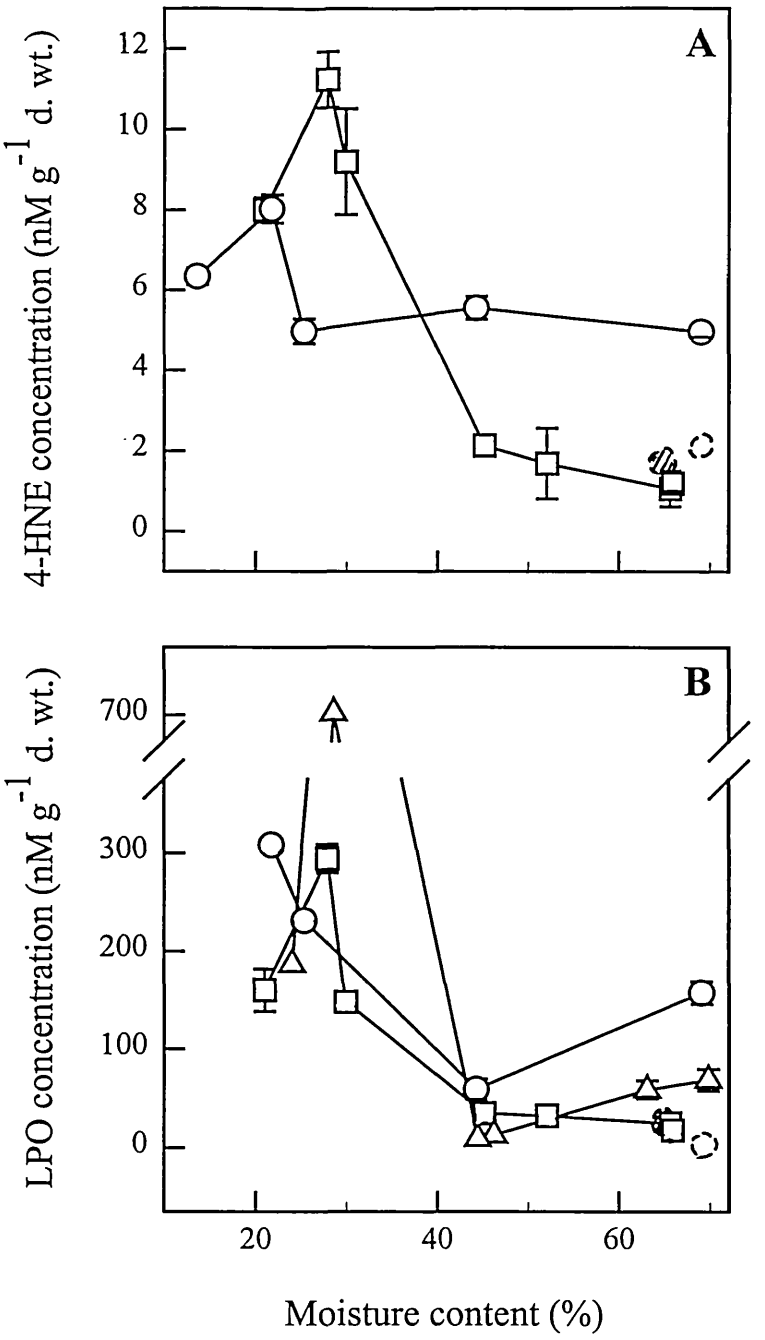


Figure 6.4

Relationship between both the 4-HNE (A) and the total LPO concentrations (B) and the moisture content of *A. hippocastanum* seed axes. Symbols are as in Fig 6.1 and bars represent s.d. Ghosted symbols represent unstored material (see Chapter 4).

the cotyledons, whereas in the axes SOD activity was slightly reduced, peroxidase activity was significantly lower (c. 6-fold) and catalase activity slightly increased (Table 6.2).

6.3.2 Effects of desiccation after hydrated storage in horse chestnut seeds

Prior to medium-term hydrated storage desiccation of batch 1 seeds over 4 d resulted in a reduction in cotyledon moisture content from c. 50 % to between c. 30 and 40 % (see Fig 4.1B); the corresponding fall in axis moisture content was from c. 70 % to between c. 15 and 35 % (Fig 4.1A). A similar pattern of moisture content reduction was evident in batch 2 seeds, and in seeds of both batches which had been stored hydrated for up to 18 months, even though the initial moisture level of the seeds varied between 50 % and 62 %. Overall, the desiccating embryonic axis of 'unstored' and stored seeds lost an average of 0.15 ± 0.01 log moisture content d^{-1} compared to a reduction of 0.06 ± 0.01 log moisture content d^{-1} at the whole seed level.

To reiterate, a reduction in seed germination during the drying of 'unstored' batch 1 seeds was first evident as embryonic axis moisture contents decreased, from around 60 % initially, to less than 40 % (Fig 4.2A). For batch 2 seed, desiccation initially increased germination, as the axis moisture content fell from 70 % to 60%, thereafter drying reduced seed germination. Thus batch 2 seeds were more desiccation sensitive than those of batch 1 such that the axis moisture content at which 50 % of the seed remained germinable was 40 %, compared to 25 % for batch 1 seed (Fig. 4.2A).

After hydrated storage for periods of 6 to 18 months, this parameter of seed desiccation intolerance always increased, to moisture contents between 45 % and 55 %. At the

Table 6.2. *Effects of desiccation on protein concentration and enzyme activity of axes extracted from* A. hippocastanum *(batch 2) seed stored hydrated at 16 °C.*

Protein and enzyme values represent the mean ± s.d. for 3 to 5 axes; triplicate determinations were made on a single extract. For the hydrated storage experiment, data are significantly different ($P < 0.05$) when followed by different letters. For desiccation experiments on stored material, data are significantly different ($P < 0.05$) when followed by different symbols.

Storage time (months)	Axis moisture content (%)	Protein concentration (mM mg^{-1} f. wt.)	Catalase activity (nmol min^{-1} mg^{-1} protein)	Peroxidase activity (nmol min^{-1} mg^{-1} protein)	SOD activity (SOD-525 units g^{-1} protein)
0	69 ± 3	1.13 ± 0.03 a *	0.12 ± 0.00 a *	29.45 ± 9.00 a *	1.28 ± 0.26 a *
0	23 ± 6	1.56 ± 0.03 +	0.63 ± 0.00 +	35.00 ± 7.60 *	1.51 ± 0.10 *
18	70 ± 1	7.64 ± 0.06 b *	1.17 ± 0.84 a *	5.00 ± 0.64 b *	1.04 ± 0.03 a *
18	24 ± 3	7.44 ± 0.96 *	0.31 ± 0.09 *	4.49 ± 0.06 *	1.04 ± 0.06 *

whole seed moisture content level, the germination of 'unstored' seed fell to 50 % after drying to moisture contents of about 35 % to 40 % (whole seed basis); hydrated storage for 6 to 18 months raised seed desiccation sensitivity to moisture contents to between 45 % and 55 % (Fig 6.5B).

Similar increases in conductivity after drying to those seen in 'unstored' seed (see Table 4.1) were observed in stored seed, although for the 18 month old seed such an increase was evident after drying to an axis moisture content of c. 44 % (Table 6.1), reflecting the elevated sensitivity to desiccation of stored seed.

Similar findings for free radical activity to that seen in freshly harvested material (see Chapter 4) were observed for seed which had been stored hydrated for 6 to 18 months prior to desiccation. Stored seeds exhibited about a doubling in the LF free radical

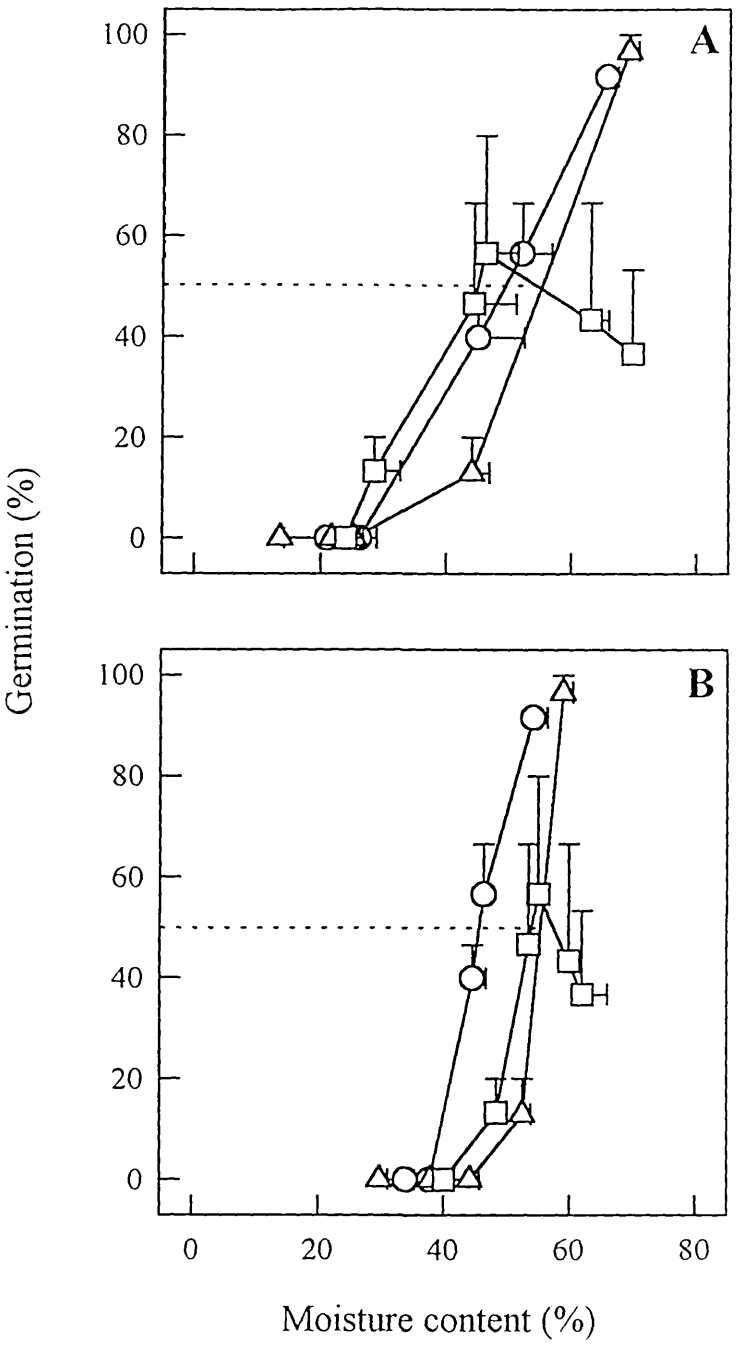


Figure 6.5

Relationship between both embryonic axis (A) and whole seed (B) moisture contents and germination of *A. hippocastanum* seeds. Symbols are as Fig 6.1, and bars represent s.d. The dotted line at 50 % germination is included as a measure of desiccation tolerance.

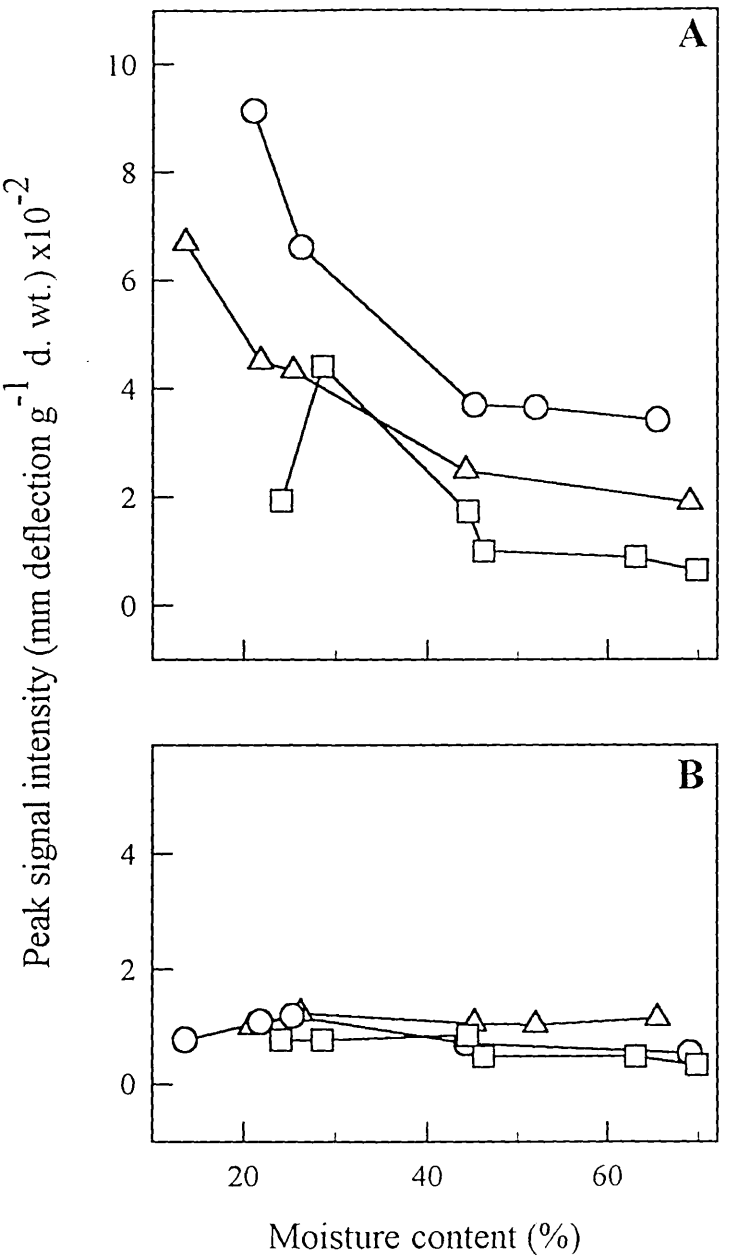


Figure 6.6

Relationship between both the low field (A) and the high field (B) EPR signal intensity and moisture content of *A. hippocastanum* seed axes. Symbols are as in Fig 6.1.

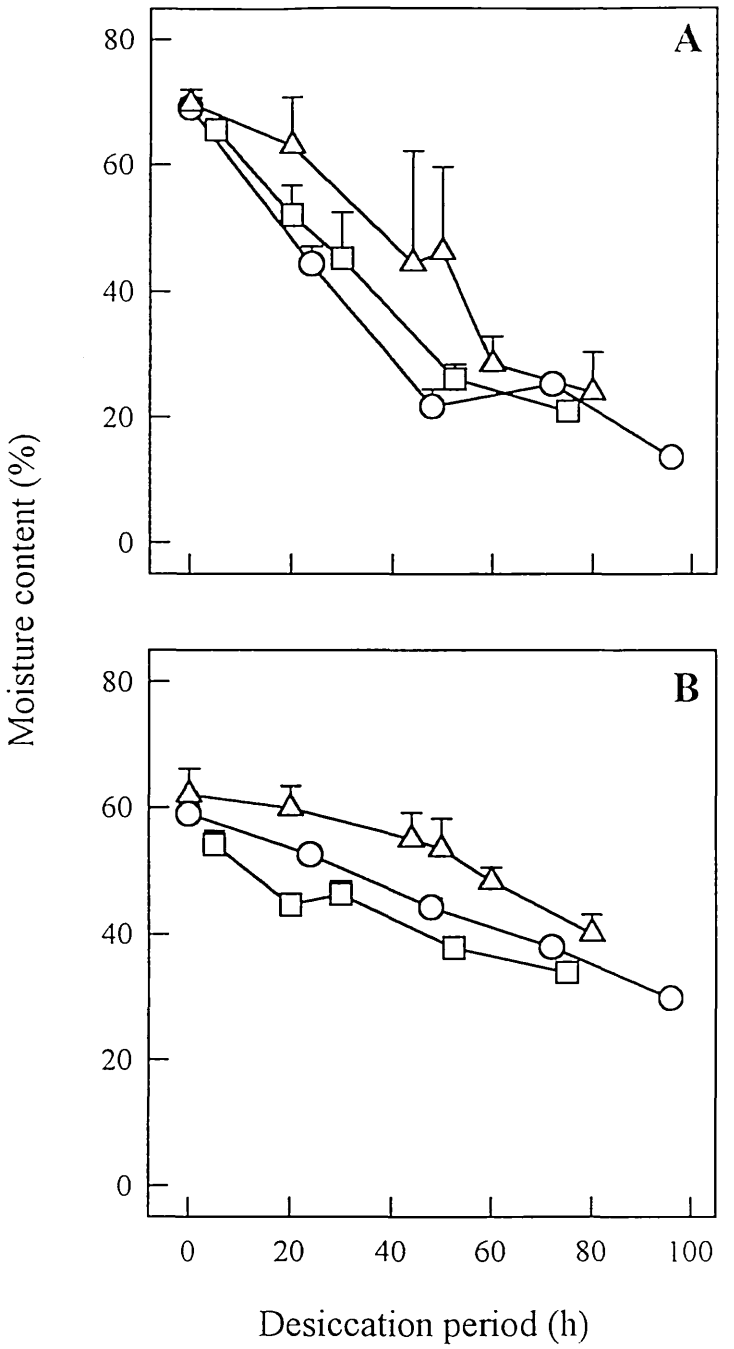


Figure 6.7

Effect of desiccation period on the embryonic axis (A) and the whole seed (B) moisture contents of *A. hippocastanum* seed. Symbols are as Fig 6.1 and bars represent s.d.

signal intensity on desiccation, but the onset for this increase occurred at an axis moisture content of ≥ 40 % (Fig 6.6A). In contrast, no significant increase ($P < 0.05$) in the HF signal was observed on drying (Fig 6.6B). As a consequence the LF : HF EPR signal intensity ratio increased to between 5 and 9, after all desiccation treatments, down to an axis moisture content of c. 25 %.

The effect of desiccation on the respiration of embryonic axes was biphasic for stored, batch 2 seed. Axes which had been dried from c. 70 % to the range 25 % to 44 % moisture content had, upon rehydration, an increased oxygen uptake of between 2 % and 47 % compared to the undried controls (Table 6.1). This increase in respiration coincided with the range of moisture contents in which most of the seed viability was lost (Fig 6.5). On further desiccation, to between 14 - 25 % axis moisture content, respiration on rehydration was reduced 4- to 7-fold lower than in the control.

The lipid peroxidation product profile of the axes following desiccation was related to viability (Figs 6.3 and 6.4). In comparison to 'unstored' material, desiccation of hydrated stored seeds generally resulted in either the accumulation of a higher concentration of specific product or an increase in the axis moisture content at which this was first observed (compare Figs 6.3 and 6.4 to Figs 4.3 and 4.4).

Desiccation did not appear to greatly affect the protein content of either the axis (Table 6.2) or the cotyledons (data not shown). Also, relatively small changes in enzyme activity in the axes accompanied desiccation of stored seeds (Table 6.2).

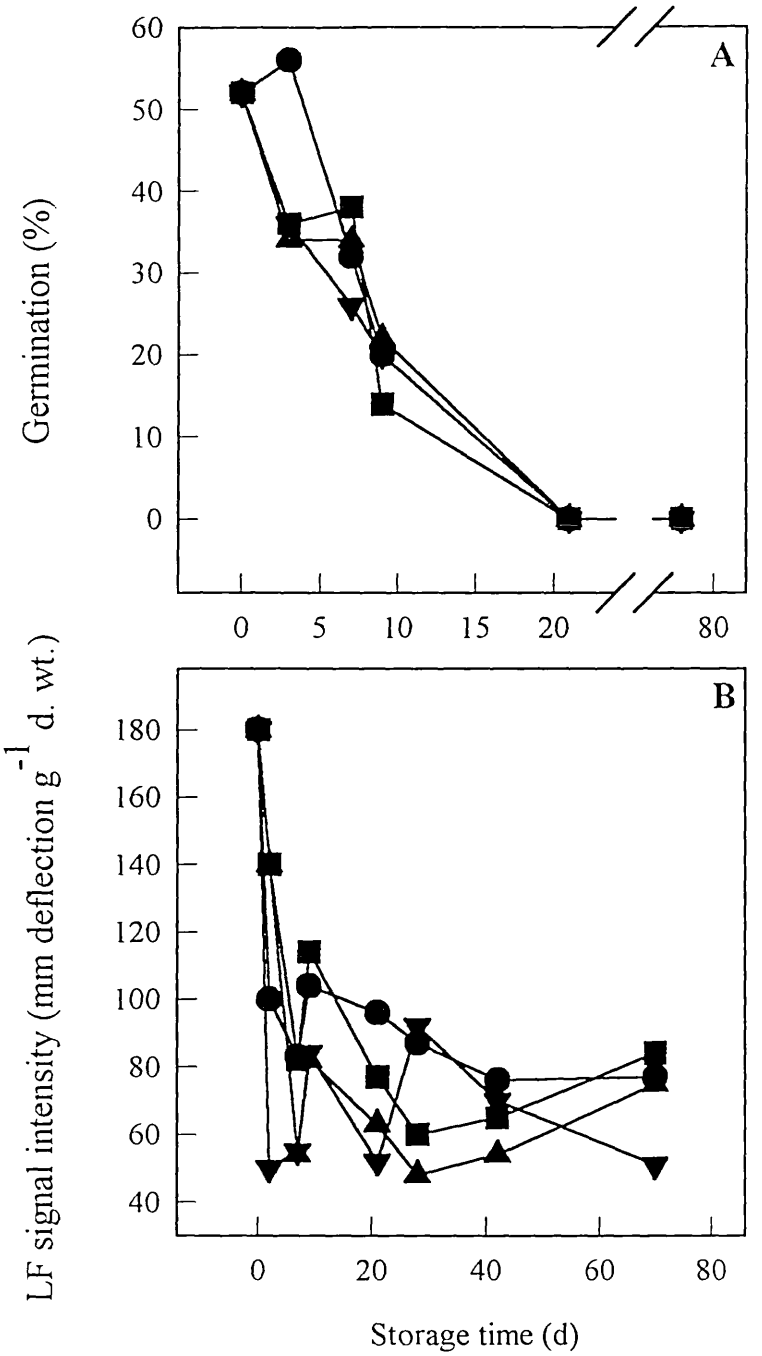


Figure 6.8

The relationship between germination (A) and LF EPR signal intensity (B) and storage time in air (circles), O_2 (squares), CO_2 (triangles) and N_2 (inverted triangles) of *Carica papaya* L. seeds. Error bars are omitted for clarity, but fell within 5 % (A) or 8 mm (B).

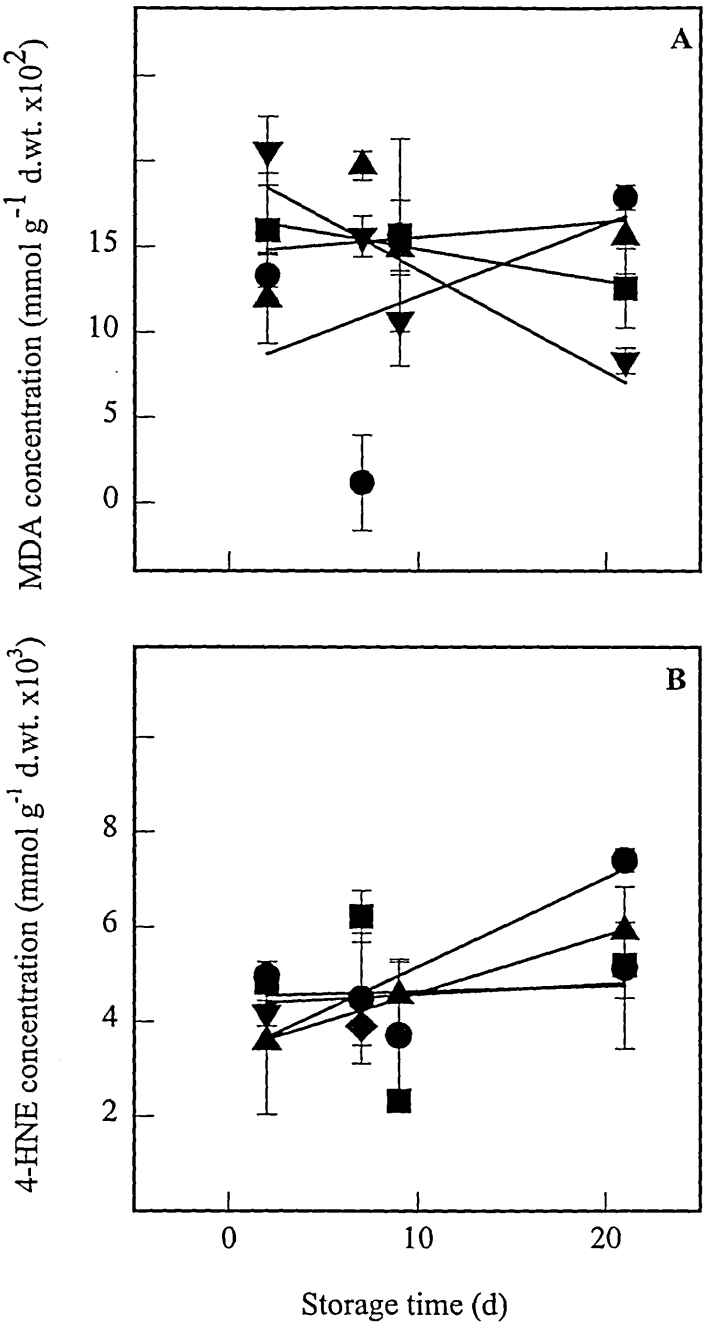


Figure 6.9 Relationship between both the MDA (A) and 4-HNE concentration (B) and storage time in differing gaseous environments of *C. papaya* L. seed. Symbols are as in Fig 6.8 and bars represent one s.d. of the mean. Lines are 1st order regressions.

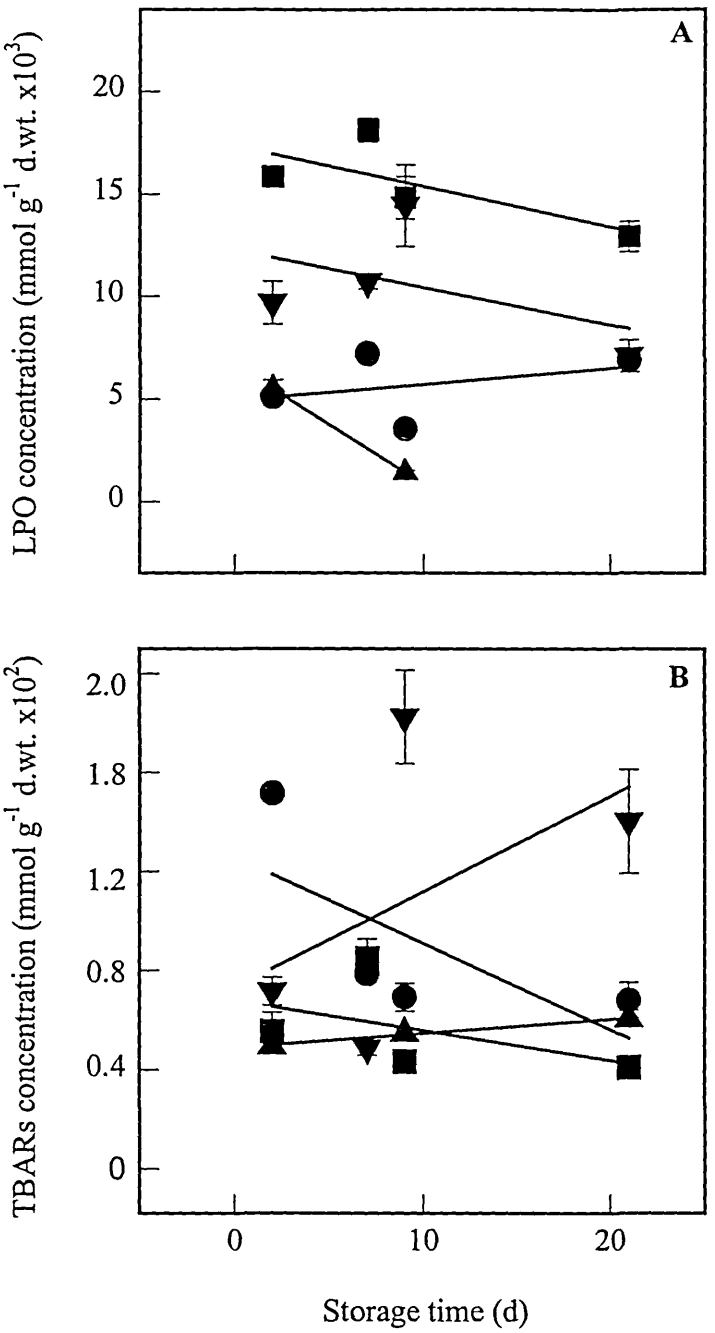


Figure 6.10 Relationship between both the total LPO (A) and TBARS concentration (B) and storage time in differing gaseous environments of *C. papaya* L. seed. Symbols are as in Fig 6.8 and bars represent one s.d. of the mean. Lines are 1st order regressions.

6.3.3 Effects of dry storage on papaya seed viability

Papaya seeds at c. 2.9 ± 0.3, 3.2 ± 0.2 and 8.2 ± 0.4 % moisture content for embryo, endosperm and testa respectively, lost viability during storage in the presence of different gases (O_2, N_2, CO_2 and air) over a 20 d period at 40 °C. Initial germination levels were 52 % (Fig 6.8A), and this decreased to between 25 and 35 % after 7 d of storage, viability loss being similar between gaseous environments. The reduction in germination was quite rapid and by 9 d the germination levels had fallen further to

Table 6.3 *Effects of dry storage at 40 °C over 72 d on lipid peroxidation product concentrations in papaya seeds (testa removed).*

Lipid peroxidation product	Treatment	Intercept (units)[1]	Slope (units d^{-1})[1]	r^2
4-HNE	Air	4181.86	41.77	0.82
	Carbon dioxide	4777.02	9.53	0.03
	Nitrogen	5344.38	9.01	0.01
	Oxygen	4152.65	49.39	0.42
MDA	Air	1187.16	5.64	0.05
	Carbon dioxide	1586.09	-1.96	0.06
	Nitrogen	1542.29	-0.17	<0.01
	Oxygen	1424.43	3.6	0.04
LPO	Air	5660.25	-6.84	0.02
	Carbon dioxide	15077.71	151.12	0.46
	Nitrogen	3800.38	-52.59	0.53
	Oxygen	13268.47	-45.19	0.02
TBARS	Air	93525.55	-175.65	0.02
	Carbon dioxide	58179.53	155.189	0.05
	Nitrogen	51872.25	-1.21	<0.01
	Oxygen	101604.39	-602.40	0.07

[1] for units see Figs 6.9 and 6.10

Table 6.4 *Results of the ANOVA investigation into the effects of different gaseous storage environments on the lipid peroxidation profiles of* Carica papaya *L. Seed (testas removed).*

A *4-Hydroxynonenal*

Storage environment: *Air*

Source	ss	d.f.	MS	F-ratio	$P =$
Regression	6012853	1	6012853	24.0	< 0.005
Error	1251012	5	250202.4		
Total	7263865	6			

Storage environment: CO_2

Source	ss	d.f.	MS	F-ratio	$P =$
Regression	312984	1	312984	0.15	0.75
Error	10784386	5	2156877.2		
Total	11097370	6			

Storage environment: N_2

Source	ss	d.f.	MS	F-ratio	$P =$
Regression	278578	1	278578	0.06	> 0.75
Error	18960992	4	4740248		
Total	19239670	5			

Storage environment: O_2

Source	ss	d.f.	MS	F-ratio	$P =$
Regression	6160931	1	6160931	2.89	0.50
Error	8512691	4	2128172.75		
Total	14673622	5			

B *Malondialdehyde*

Storage environment: *Air*

Source	ss	d.f.	MS	F-ratio	$P =$
Regression	234787	1	234787	0.53	0.75
Error	1785972	4	446493		
Total	2020759	5			

Storage environment: CO_2

Source	ss	d.f.	MS	F-ratio	$P =$
Regression	11679	1	11679	0.25	0.75
Error	184927	4	46231.75		
Total	196606	5			

Storage environment: N_2

Source	ss	d.f.	MS	F-ratio	$P =$
Regression	99	1	99	0.001	> 0.75
Error	406669	5	81333.8		
Total	406768	6			

Storage environment: O_2

Source	ss	d.f.	MS	F-ratio	$P =$
Regression	44741	1	44741	0.53	0.50
Error	1052573	5	210514		
Total	1097314	6			

C *Total Lipid Peroxides*

Storage environment: *Air*

Source	ss	d.f.	MS	F-ratio	$P =$
Regression	161323	1	161323	0.09	> 0.75
Error	9358150	5	1871630		
Total	9519473	6			

Storage environment: CO_2

Source	ss	d.f.	MS	F-ratio	$P =$
Regression	78698272	1	78698272	0.42	0.75
Error	93039936	5	186079867		
Total	171738208	6			

Storage environment: N_2

Source	ss	d.f.	MS	F-ratio	$P =$
Regression	7741961	1	7741961	2.23	0.50
Error	6945022	2	3472511		
Total	14686983	3			

Storage environment: O_2

Source	ss	d.f.	MS	F-ratio	$P =$
Regression	7040128	1	7040128	0.09	> 0.75
Error	408105600	5	81621120		
Total	415145728	6			

D *Thiobarbituric reactive substances (TBARS)*

Storage environment: *Air*

Source	ss	d.f.	MS	F-ratio	$P =$
Regression	101721600	1	101721600	0.09	> 0.75
Error	5554213888	5	111084277		
Total	5655935488	6			

Storage environment: CO_2

Source	ss	d.f.	MS	F-ratio	$P =$
Regression	1250311170	1	125031170	0.37	0.75
Error	16573074432	5	3314614886		
Total	17823385600	6			

Storage environment: N_2

Source	ss	d.f.	MS	F-ratio	$P =$
Regression	82979840	1	82979840	0.27	0.75
Error	1516749184	5	303349836.8		
Total	1599729024	6			

Storage environment: O_2

Source	ss	d.f.	MS	F-ratio	$P =$
Regression	- 4096	1	4096	$3.1e^{-6}$	> 0.75
Error	5251513856	4	131287464		
Total	5251517952	5			

between 13 and 22 % respectively. From day 21 onwards there was a total loss of viability (Fig 6.8A).

With storage there was also a decline in the overall EPR signal intensity values for all treatments (Fig 6.8B), values falling from c. 180 to between c. 80 and 50 mm deflection g^{-1} d. wt over the 72 d storage period. However, the loss of free radical signal intensity was not uniform, with a considerable amount of variation between 0 to 20 d of storage. Statistical analysis revealed that although there was a significant reduction in EPR signal intensity ($P < 0.10$), there was no effect of gaseous environment upon this response ($P < 0.01$).

Results from lipid peroxidation studies were also very variable, with no immediate trends apparent (Figs 6.9 and 6.10). A simple first order regression yielded low correlation coefficients (≤ 0.53), except for 4-HNE which increased in air storage, $r^2 = 0.82$ (Figs 6.9, 6.10 and Table 6.3). Further statistical analysis (Table 6.4), using a one-way ANOVA test, confirmed that only 4-HNE in the air treatment increased during storage.

6.4 Discussion

6.4.1 Effects of hydrated storage

Seeds of horse chestnut are shed in a relatively immature state, at high embryonic axis moisture contents (Table 6.1, Fig 6.5) equivalent to a water potential of about -1.1 MPa (Tompsett and Pritchard, 1993). The axes of such seeds are metabolically active (Table 6.1), contain numerous lipid peroxidation products (Figs 6.3 and 6.4) and have

relatively low enzyme activity against activated oxygen (Table 6.2). In addition, even when the germination capacity of the seeds is high (Fig 6.1, Table 6.1), the axes consistently contain free radicals, as determined by EPR (Fig 6.6). The free radical signal observed in non-senescent axes of both fresh (Chapter 4) and stored horse chestnut seeds consisted of two peaks which differed in signal intensity (Fig 3.4). This is in contrast to the earlier findings of Hendry and co-workers who noted only a single, LF signal in wet, viable axes of horse chestnut and two other recalcitrant species (Hendry *et al.*, 1992 and Finch-Savage *et al.*, 1994). As previously mentioned (Chapter 3), type III water quenching of the EPR signal is associated with measurements being made at ambient temperatures, and the resolution of two peaks here (Fig 3.4) is most likely due to the 'freezing out' of this effect by making measurements at -196 °C.

Hydrated storage of horse chestnut seeds was achieved using the same agar-water medium as that used for routine germination testing. During storage the seeds rehydrated beyond their shedding moisture content and we estimate from an isotherm published previously (Tompsett and Pritchard, 1993) that the axes were at a water potential during storage of > -1 MPa and thus probably contained type V water (Vertucci and Farrant, 1995). At such an elevated water potential, germination would have undoubtedly occurred at conducive temperatures (Fig 6.1; Pritchard *et al.*, 1996). The fact that seeds neither increased in axis dry weight nor pre-sprouted during storage at 16 °C indicates that the seeds were inhibited from germinating by temperature limitation rather than through the imposition of desiccation stress. The value of using minimum temperatures for germination to enhance recalcitrant seed storage has also been demonstrated for seeds of *Araucaria hunsteinii* K. Schum. (Pritchard *et al.*, 1995b).

Continued respiratory capability, an increased protein content of the axes (Table 6.1) and an increase in free radicals as perceived by an elevated signal in both the LF and HF region (Fig 6.6) indicate that metabolism continues unabated under hydrated storage conditions. The increased germination rate at 35 °C by 6 months of hydrated storage (Fig 6.1) suggests that such metabolism may have been associated initially with the continuing development of the seeds post-harvest, as earlier reported for this (Tompsett and Pritchard, 1993) and another recalcitrant-seeded species (Farrant *et al.*, 1993). However, longer term increases in membrane leakage and some lipid peroxidation products (Table 6.1, Figs 6.3 and 6.4) indicate that other less advantageous aspects of oxidative metabolism were also in progress and that these probably contributed to the slow loss of viability during storage (Fig 6.1; Pritchard *et al.*, 1996). Similarly declining membrane integrity, as a result of oxidative stress, has been observed in potato seed-tubers stored for up to 32 months (Kumar and Knowles, 1993). The role and identity of the free radical(s) involved in such natural ageing remains unknown, but transient increases in the superoxide radical are known to accompany the relatively long-term (weeks) senescence of bean leaves (Thompson *et al.*, 1987). In horse chestnut seeds, failure to compensate for an increased presence of the superoxide free radical with enhanced SOD activity (Table 6.2) could have contributed to an increase in oxidative stress. Moreover, the increased presence after 12 months storage of 4-HNE, an aldehydic product of peroxidation, may be particularly significant as a potentiating step in the final stages of viability loss; studies on animal systems have implicated this chemical in the loss of both biochemical and genetic function (Zollner *et al.*, 1991).

As with other recalcitrant seed axes (Hendry *et al.*, 1992), enzyme activity against activated oxygen is relatively low in horse chestnut axes at the start of storage and, for peroxidase at least, decreases with storage time as viability is being lost (Fig 6.1, Table 6.2). Interestingly, reduced levels of peroxidases, and the accumulation of lipid peroxidation products, occur in suspension culture cell lines which have lost the ability for embryogenesis (Benson *et al.*, 1992). The similarities in the biochemistry of the loss of morphogenic potential (germination) in recalcitrant horse chestnut seeds and the embryogenic potential of suspension culture cells emphasises that oxidative stress can modulate cell growth, even under non-desiccating conditions. Therefore, although horse chestnut seeds have a considerable longevity in hydrated storage at warm temperatures (Fig 6.1; Pritchard *et al.*, 1996), the seeds appear to eventually succumb to the slow process of oxidative stress under conditions of full hydration.

6.4.2 Effects of desiccation after hydrated storage

The observed sensitivity of stored horse chestnut seed axes to drying below about 40 % moisture content clarifies the earlier finding that horse chestnut seeds are recalcitrant (Chapter 4; Tompsett and Pritchard, 1993) with an onset for desiccation stress of approximately - 4 MPa, similar to the axes of numerous other recalcitrant seeds (Pritchard, 1991; Poulsen and Eriksen, 1992; Pritchard *et al.*, 1995a and Vertucci and Farrant, 1995). Not only do such seeds exhibit little tolerance to drying (Fig 6.5) they have no particular enhanced ability to resist desiccation either (Figs 4.1 and 6.7). The complete loss of axis viability in horse chestnut by c. 25 % moisture content (c. -25 MPa) is consistent with observations on other desiccating recalcitrant seeds (Pritchard, 1991, Poulsen and Eriksen, 1992, Pritchard *et al.*, 1995a and b; Vertucci and Farrant, 1995) and supports the view that the removal of hydration level III water maybe

particularly critical to the survival of truly-recalcitrant seed (Chapter 5; Vertucci and Farrant, 1995).

The precise mechanism of desiccation injury remains unknown. However, the involvement of secondary, oxidative, stress fuelled by an unregulated respiratory metabolism appears likely (see previous Chapters), as previously proposed for two model 'recalcitrant' seed systems, immature and germinating orthodox seeds (Leprince *et al.*, 1992, 1993 and 1994). In recalcitrant horse chestnut, the level of oxidative stress increases dramatically, and coincidentally with viability loss, when the axes are dried from 44 % to 25 % moisture content; respiratory activity increases (Table 6.2), membrane integrity is compromised (Table 6.2) and lipid peroxidation products (Figs 6.3 and 6.4) and free radicals accumulate (Fig 6.6). Similarly, respiratory activity was not impaired during the early stages of desiccation-induced viability loss in embryo tissues of *Quercus robur* (Poulsen and Eriksen, 1992) and *Araucaria angustifolia* (Bert.) O. Ktze. (Espindola et al., 1994). Moreover, the absence of any promotive effect of desiccation on the activity of antioxidant enzymes (Table 6.2), may contribute to an enhanced level of oxidative activity.

In contrast to the earlier work on desiccating recalcitrant seed axes (Hendry et al., 1992 and Finch-Savage et al., 1994 and 1996), there was no evidence that the free radical(s) which contribute to the LF EPR signal accumulated prior to dehydration-induced viability loss in axes of horse chestnut (compare Figs 4.5 and 6.6). Also, my results do not support the hypothesis that the HF EPR signal has physiological significance during desiccation-induced viability loss as suggested by Hendry et al (1992) as the intensity of this signal was independent of moisture content in horse chestnut axes (Fig 6.6).

Significant differences between the oxidative stress components of natural ageing (see earlier) and viability loss caused by desiccation lead me to believe that there are a number of free radical species involved in the death of recalcitrant seeds of which a stable quinone-derived free radical may be but one component (see Atherton *et al.*, 1993). Firstly, the relative intensity of the LF peaks varied with moisture content whilst that of the HF peak did not (Fig 6.6), indicating the presence of more than one free radical species. Secondly, natural ageing (hydrated storage) led to a much smaller and transient increase in both the LF *and* HF EPR signal. Also, the different intensity changes in the LF signal, the lipid peroxidation product profile (see later discussion) and the membrane leakage rates under the two types of stress applied in this study highlight the complexity of the oxidative stress response in recalcitrant seed material.

Desiccation after storage clearly resulted in an accumulation of lipid peroxidation products and a build up of free radical signal intensity. However, the moisture content at which injury was evident was raised by c. 15 % after hydrated storage. It was also apparent that upon desiccation the embryonic axes underwent a higher degree of lipid peroxidation and free radical accumulation than that of freshly harvested material (compare Figs 6.3 and 6.4 with 4.3 and 4.4).

Significant increases in electrolyte leakage (c. 30 %) were also noticeable upon desiccation, indicating that the cells within the embryonic axes had undergone massive membrane perturbations. This is in contrast to what I observed when the seeds were stored hydrated prior to desiccation. It seems clear therefore that the processes which result in a reduction in viability upon storage are not the same as those when the seed is

desiccated (either before or after storage). Desiccation is manifested as a gross disruption to membrane integrity (see previous Chapters).

These findings can be attributed to events of oxidative stress occurring at critical moisture contents, which finally result in the seeds loss of viability. In stored seed the respiration rate upon viability loss increased by approximately 3 times that of 'unstored' material, indicating that at the point of viability loss stored seed has a greater oxidative component than desiccating non-stored seed. Thus, a corollary of the above suggests a close relationship exists between respiratory activity, storage metabolism and free radical mediated damage.

I postulate therefore, that the effect of prolonged hydrated storage of desiccation sensitive seeds is a slow accumulation of respiratory products, which result in a greater, oxidative response during desiccation stress. The respiratory measurements support this hypothesis, both freshly harvested and stored material had their respiratory activity revoked at very low moisture contents (when the seed had lost viability). However, at the point of viability loss, respiration increased slightly, this mirrors the peak of activity we see in the free radical and lipid peroxidation measurements.

6.4.3 Effects of dry storage

King and Roberts (1979) suggested that the relationship of decreasing longevity with increasing moisture content, indicated by the viability equation may reverse abruptly at, or close to c. 90 % eRH (Roberts and Ellis, 1989 and Vertucci, 1989). One difficulty in understanding such relationships lies, however, with the fact that in most previous work (*save* Ibrahim and Roberts, 1983) these considerations have always been made under

normal atmospheric conditions. However, many dry seeds are invariably stored in hermetically sealed containers (in seed banks ect.). Under these conditions the partial oxygen pressure decreases with time, due to seed respiration and / or gaseous exchange (Roberts and Abdalla, 1968). It has been argued that higher oxygen concentrations tend to decrease slightly the longevity of dry seeds (Roberts 1972). However Ibrahim and Roberts (1983) argue that in moist seeds oxygen may be beneficial as it is invariably involved in repair and turnover mechanisms, this change in role appears to be controlled by crossing a certain critical moisture content.

Ibrahim and Roberts (1983) work suggests that there is an interaction between moisture content and oxygen concentration, they also show that there appears to be a close relationship between moisture content, turnover rate and repair mechanisms in hydrated seed (which begin to operate at ≥ 15% moisture content in lettuce seeds), which is governed by oxygen concentration. Seed stored under anaerobic conditions are incapable of sustained repair, and viability is lost quickly. However, under aerobic conditions the rate of normal metabolism increases with moisture content, sustained by respiration so that repair and turnover are favoured. It was also postulated that this process is temperature dependent, so that at higher temperatures survival is shortened.

It is of course possible that the deleterious effects of anaerobic conditions on moist seed could be due to the accumulation of fermentation products. However, Tompsett (1983) has stored moist seeds of *Araucaria hunsteinii* (a recalcitrant tropical) in which viability loss is rapid under anaerobic conditions. and saw no evidence of ethanol accumulation He attributed this response again to an interaction between moisture content,

temperature and oxygen concentration. It is clear therefore that oxygen concentrations may affect the viability of hermetically stored seeds.

Magill *et al.*, (1994) have revealed that oxidative metabolism is reduced during papaya seed desiccation, and that this is a process that does not affect seed survival. I wanted to determine the influence of oxygen concentration on the survival of stored papaya seed, and examine the role of oxygen metabolism during this process.

The requirement of oxygen for seed survival has been studied on a number of occasions (Bewley and Black, 1982, Côme, 1982, Mayer and Piljakoff-Mayber, 1989 and Côme and Corbineau, 1992). However, it has not always been clear whether the results of such experiments are related to their oxygen sensitivity or dormancy effects. The germinability of seed may also be related to other environmental interactions such as temperature (Corbineau and Côme, 1995 and above). For this reason it is important to fully understand the germination requirements, and dormancy status of material to be used for an investigation of this type. A detailed study was therefore made of the germination requirements of papaya seed (Chapter 7), in order to accurately reflect the effects of gaseous storage (and hence oxygen concentrations) on the viability loss of this species.

Depending upon structure seed coats are more or less permeable to gases in the dry state (Côme, 1970 and 1982). However, imbibed seed coats can limit gaseous diffusion since the water forms a continuous obstructive layer around the embryo, in which gases can dissolve (Côme, 1970 and 1982). Further more, coats of numerous seeds contain high concentration of polyphenolics (Bewley and Black, 1982 and Côme, 1982), the

oxidation of which by polyphenolic oxidases further reduces oxygen concentrations surrounding the seed embryo (Côme, 1970 and 1982). In such seeds these effect also increase with temperature, since gases become less soluble, and the oxidation of phenolics is more intense. To ensure that this would not affect the investigation of hermetically stored papaya seed, the seeds were equilibrated to low moisture contents before storage. During storage the moisture content of the seeds was continually monitored, to ensure that there were no extraneous effects related to changes in hydration level.

The rate of viability loss in stored papaya seeds was found to be similar for all treatments, independent of the gas used and therefore relative oxygen concentration. Therefore, in papaya at least, it does not seem that the rate of viability loss during storage at elevated temperatures is necessarily related to oxygen concentration. This is in contradiction to the work carried out by Ibrahim and Roberts (1983) and Tompsett (1983) using both orthodox and recalcitrant material.

Villiers and Edgcome (1975) suggested that viability loss of dry-stored orthodox seeds may be related to subcellular deterioration, and this would be dependent upon both temperature and moisture content. However, the high storage temperature employed in this investigation, permits normal metabolism to occur (Ibrahim and Roberts, 1983), and suggests that there may be a continual operation of repair mechanisms, helping to maintain cellular integrity. Conversely, the operation of such systems may be ineffectual if seed deterioration has progressed beyond the point of repair. In this situation a culmination of cellular damage would eventually lead to the loss in seed viability observed. Ibrahim and Roberts (1983) argue that the operation of such repair systems is

regulated by, and dependent upon oxygen, they also present evidence that such mechanisms may begin to operate at c. 15 % moisture content. The loss of viability of papaya seeds stored under differing oxygen concentrations would argue against such a hypothesis, however the moisture content to which papaya seed hydrates at 75% eRH is below the 15 % operation limit. This may explain the loss of viability during storage, the full effects of repair not apparent unless the seeds are hydrated further.

There is, however, a clear relationship between viability loss, desiccation and oxidative metabolism in recalcitrant seeds (this and earlier chapters), a relationship which is not true of desiccation-tolerant (see Chapter 7) seeds (Magill *et el.*, 1994; using papaya). It is not surprising therefore that for papaya seeds there was no clear relationship between relative oxygen concentration and viability loss during dry-storage.

Moreover, it seems clear that the effects upon oxidative metabolism observed in recalcitrant seeds during desiccation, do not occur in desiccation-tolerant dry material (see Chapter 7). This contrast in oxidative response is also apparent during storage, hydrated storage of recalcitrant horse chestnut seeds, serves to exacerbate the effects of a highly reactive oxygen metabolism. However, oxygen concentrations have no effect on viability loss of papaya seeds during dry storage. Not only is the immediate environmental oxygen concentration non-contributory, but also the oxidative metabolism of the seed appears to play no significant role in the loss of viability.

In conclusion, one may hypothesise from these results that the inability of recalcitrant seeds to survive desiccation, may be related to the uncontrolled increases in oxidative metabolism that occur during drying, an effect which is also metabolically modulated

during storage. The lack of an oxidative response to drying in desiccation-tolerant material, may therefore explain their capacity to maintain viability upon desiccation (see Chapter 8).

6.5 Summary

Low temperature hydrated storage of horse chestnut seeds limits germinability, but does not deleteriously alter metabolic activity. The continuing activity of physiological and biophysical cellular events may be associated with post-harvest development, however, there were also signs of a gradual loss of viability upon hydrated storage. Measurements of oxidative metabolism and antioxidant capacity also reveal that oxygen related stress may have a modulating effect upon cell development during hydrated storage.

Upon storage desiccation sensitivity increased, reflected by a quicker, larger increase in lipid peroxidation, compared to non-stored material. However, the mechanisms of viability loss that occur during desiccation and storage are not necessarily related. It seems therefore, that oxidative stress may serve as a diagnostic marker for seed storage behaviour.

This is supported by the finding that desiccation-tolerant (Chapter 7) papaya seed displays a lack of oxidative activity upon drying and storage. Moreover, the loss of viability in stored papaya seeds has no relation to either its immediate surrounding oxygen concentration, or the endogenous oxidative metabolism of the seed.

CHAPTER 7

A Physiological Investigation of Storage and Germination Parameters of a Desiccation-tolerant Seed Species - *Carica papaya* L.

Chapter 7: A physiological investigation of storage and germination parameters of a desiccation-tolerant seed species - *Carica papaya* L.

Outlay of Chapter

Chapter 7: A Physiological Investigation of Storage and Germination Parameters of a Desiccation-tolerant Seed Species - *Carica papaya* L.

7.1 Introduction

Unlike desiccation-sensitive seeds, the seeds of some species can be routinely dried to low moisture contents. This enables seeds of many species to be stored for long periods of time at sub-zero temperatures whilst retaining viability. However, some seeds are desiccation tolerant within limits, and their tolerance of frozen (-20 °C) or chilled (0 °C) storage is highly variable (Ellis *et al.*, 1990 and 1991a). This type of material has been described as 'intermediate' in seed storage response. The classification of seeds into broad storage categories has, in the past, been complicated by changes in dormancy status post-desiccation, meaning that the optimal conditions for viability testing have altered (Roberts *et al.*, 1984 and Kovach and Bradford, 1992).

Activated oxygen and / or its products have been shown to cause oxidative injury in a number of recalcitrant species (Hendry *et* al., 1992, Finch-Savage *et* al., 1994 and previous chapters). However, oxygen is essential for the germination of many seeds (Baskin and Baskin, 1998), it is possible therefore, that a relationship exists between germination, respiration rate and the relative desiccation sensitivity of seeds (Leprince *et al.*, 1994).

In order to see if such associations apply and to further determine their possible effects as to seed storage classification, the germination characteristics of *Carica*

papaya L. (papaya) seeds have been investigated. Because little is known about the dormancy release mechanisms of papaya seed, this approach should lead to an increased understanding of the role of oxygen in the germination process, and enable the development of a routine germination technique for this species. Such studies are also needed because dormancy may confound the interpretation of seed desiccation studies leading to the miss-classification of seed storage type e.g. *Zizania palustris* L. (Kovach and Bradford, 1992).

Papaya seed was chosen as a model system because although the fruit has been identified as producing intermediate seeds (Ellis *et al.*, 1991a), they have been shown to germinate erratically after drying (Ellis *et* al., 1991b and Magill, 1994). The germination of papaya seed was therefore studied in detail, and used to determine the sensitivity of the seed germination process to oxygen, pre-desiccation and frozen dry storage. The objective was to establish whether papaya seeds really are intermediate, and whether dormancy confounds the response to desiccation.

7.2 Experimental design

7.2.1 Seed procurement and aspects of germination

Fruits of papaya (var. 'solo') of varying origin were purchased commercially (see Table 2.1; batches 1 to 3) and were stored until use as described in Table 2.2. Upon extraction from the fruit, seeds were carefully blotted using absorbent paper to remove the fleshy sarcotesta, and then cleaned by rinsing briefly in distilled water. Physiological characteristics of the fruit and seed were noted, and subsequent germination testing was

achieved by placing two replicates of 25 seeds per treatment on 1% agar water in 9 cm Petri dishes. Initial tests were performed at 26 °C in the light (12 h d^{-1}). Later, an alternating temperature (Ellis *et* al., 1990) regime of 33 / 19 °C was used (as these were the closest conditions available to those used by Ellis *et al.*, i.e. 30 / 20 °C). A higher temperature of 36 °C was also investigated. Germination was recorded when radicle extension was ≥ 5 mm.

In some cases, mainly after desiccation, viability was checked using tetrazolium salt (TZ) staining. Embryos were dissected from seeds, and rehydrated on 1 % agar water for 1 d at 21 °C. They were then left in cold (2 °C) TZ solution, in the dark, for 24 h, before being incubated at 26 °C for a further 24 h to allow the stain to develop. The intensity of the red staining related to the viability of the material, the more intense the staining, the higher the viability. The development of an improved method of germination using a heat shock treatment is described in the results.

Uptake or evolution of gases by papaya seeds during germination was measured using the Gilson single valve differential respirometer as previously described (chapter 2). Seeds were rehydrated on 1 % agar water, and pre-incubated at 26 °C for 5 d. Subsequently, respiration was measured using five seeds in each of three replicate flasks per treatment. Oxygen uptake was recorded every 10 mins during all phases of germination.

A study of papaya seed germination in different gaseous environments was achieved by rehydration and incubation of seeds (n = 2 x 25) on 1 % agar water in Oxoid HP11 gas jars (Oxoid Ltd, Basingstoke, U.K.), containing either 100 % O_2, CO_2, N_2, or air.

Oxygen-free environments were maintained using low temperature anaerobic catalysts (BR42; Oxoid Ltd, Basingstoke, U.K.), and conditions of anoxia were confirmed by 'Resazurin' indicator strips (BR55; Oxoid Ltd, Basingstoke, U.K.). Gaseous pressure was maintained at 1 atm by repressurising the jars every 2 - 3 d.

7.2.2 Drying and freezing treatments

Freshly isolated seeds were desiccated in the dry-room for various lengths of time from 0 to 312 h. Samples were withdrawn at regular intervals and assessments made of the seed moisture content (embryo, mesotesta and endosperm), % eRH (whole seed) and germination level.

Some dried seeds (n = 25) were also transferred to laminated foil bags, that were hermetically sealed on all sides. The bags, in duplicate, were subsequently placed at a range of temperatures from 16 to -196 °C for a 1 d period. The bags were then rewarmed on the lab bench before seeds were removed and sown for germination. Treated seeds were directly rehydrated on 1% agar water as part of the germination tests.

7.3 Results

7.3.1 Physiological parameters and effects of desiccation

Fresh fruits were mostly orange, and seeds were black; thus, a physical index of 'maturity' suggested only a small amount of variation in developmental age. The general appearance and number of seeds found within batch 1 and 2 fruits is recorded in Table 7.1. Prior to treatments whole fruit moisture contents varied from 4.1 ± 1.3 to 9.5

± 2.8, 10.6 ± 3.8 to 26.2 ± 15.6 and 59.9 ± 2.1 to 69.1 ± 3.8 for the seed embryo, endosperm and testa respectively. The position of the seed in the fruit did not appear to have an effect on seed moisture content, along the longitudinal axis of the fruit the average tissue moisture contents were 4.0 ± 1.3, 9.0 ± 2.0 and 60.8 ± 2.4 for the seed embryo, endosperm and mesotesta respectively.

Placing seeds in the dry room resulted in a rapid decline in seed moisture content. Within c. 24 h moisture contents had fallen to c. 7, 8.5 and 10% respectively for seed embryo, endosperm and testa respectively (Fig 7.1).

Initial germination at constant temperatures in the light (12 h photoperiod) of batch 1 and 2 seed resulted in c. 80, 30 and 100 % of the seeds germinating at 26, 36 and an alternating temperature of 33 / 19 °C respectively (Fig 7.2). However, the ability of the seeds to germinate was generally lost when the seeds were dried to less than 10 % total moisture content (equivalent to 2 d drying; see Figs 7.1 and 7.2). This effect was not immediate, as germination at 26 °C decreased from day 1 onwards, but the moisture content between day 1 and 13 did not significantly alter ($P < 0.05$; Fig 7.1). Also, seeds which failed to germinate at 26 °C, germinated when they were transferred to 33 / 19 °C. Thus, 33 / 19 °C appeared to be a more suitable temperature for germination after a desiccation treatment had been applied (Fig 7.2). While germination at constant 26 °C was satisfactory for fresh, undried seed.

Table 7.1
Variation in fruit and seed quality parameters.

Fruit	Fruit colour	Fruit firmness	Fruit flesh colour	Seed colour	Number of seeds in fruit
1	yellow	soft	orange/pink	black	304
2	yellow	soft	orange/pink	black	228
3	yellow	firm	orange/pink	black	191
4	yellow	firm	orange/pink	black	324
5	yellow	soft	orange/pink	black	403
6	yellow/green	soft	orange/pink	black	199
7	yellow/green	soft	orange/pink	black	408
8	yellow/green	soft	orange/pink	black	290
9	yellow	soft	orange/pink	black	410
10	yellow/green	firm	orange/pink	black	360
11	yellow/green	hard	orange/pink	black	251
12	yellow/green	hard	orange/pink	black	376
13	yellow/green	hard	orange/pink	black	334
14	yellow/green	hard	orange/pink	black	395
15	yellow/green	hard	orange/pink	black	378
16	yellow/green	hard	orange/pink	black	388
17	yellow/green	hard	orange/pink	black	466
18	yellow/green	firm	orange/pink	black	430
19	yellow	hard	orange/pink	black	625
20	green	hard	orange/pink	black	296
21	yellow	soft	orange/pink	black	265
22	green	hard	orange/pink	black	561
23	green	firm	orange/pink	black	340
24	yellow/green	firm	orange/pink	black	266
25	yellow/green	firm	orange/pink	black	293
26	yellow/green	soft	orange/pink	black	458
27	yellow/green	firm	orange/pink	black	337
28	green	hard	pale orange	black	419
29	yellow	hard	orange/pink	black	408
30	yellow	soft	orange/pink	black	322
31	yellow/green	firm	orange/pink	black	378
32	yellow	soft	orange/pink	black	342
33	green	hard	pale orange	black	447
34	yellow/green	firm	orange/pink	black	389
35	yellow	firm	orange/pink	black	591
36	green	firm	orange/pink	black	596
37	yellow	firm	orange/pink	black	508
38	yellow	soft	orange/pink	black	674
39	yellow	soft	orange/pink	black	487
40	yellow/green	firm	orange/pink	black	585
41	yellow	soft	orange/pink	black	709
42	yellow	soft	orange/pink	black	481
43	yellow	soft	orange/pink	black	446
44	green	hard	pale orange	black	606
45	yellow	soft	orange/pink	black	468
46	yellow	soft	orange/pink	black	436

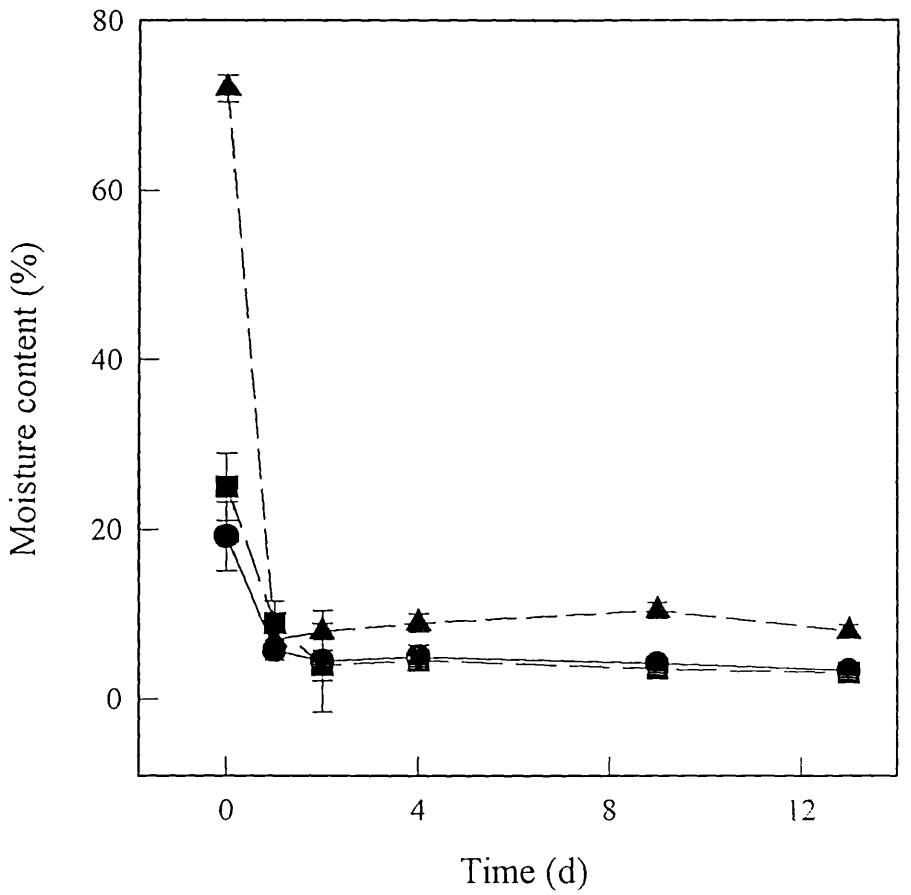


Figure 7.1

Relationship between the average moisture content of batch 1 and 2 *Carica papaya* L. seed and desiccation time for the embryo (circles), endosperm (squares) and mesotesta (triangles). Error bars represent one s.d. of the mean.

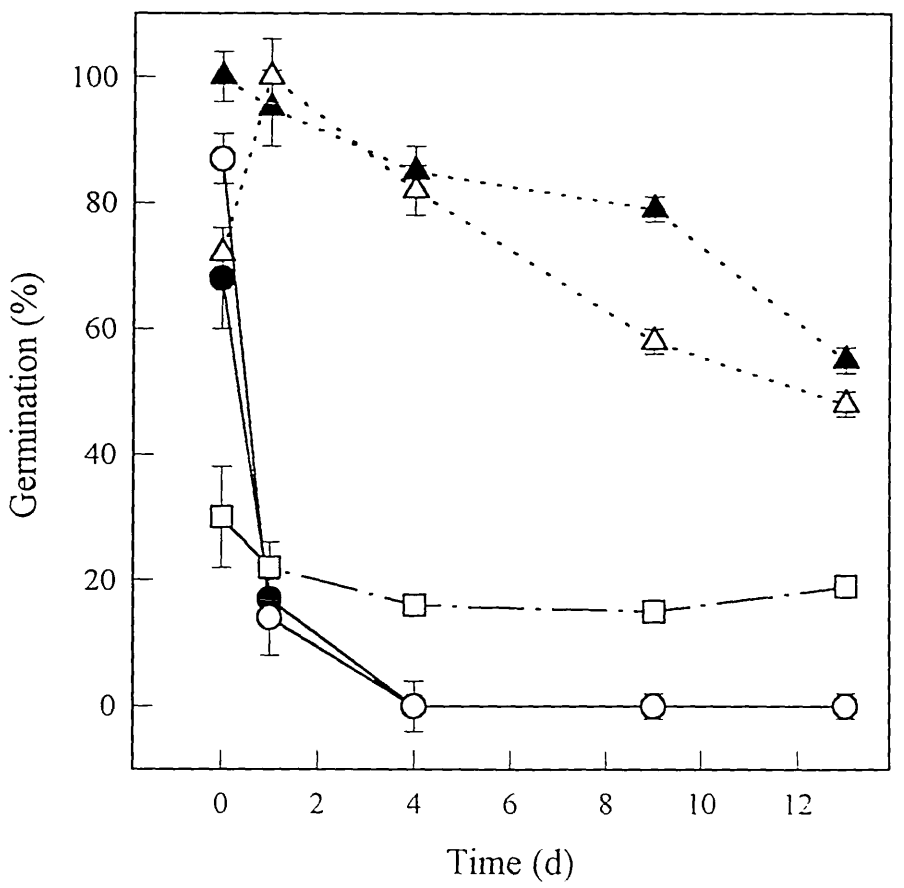


Figure 7.2

Effect of desiccation on the germination of *Carica papaya* L. seeds germinated at 26 °C (circles), 36 °C (squares) and 33 / 19 °C (triangles). Open symbols represent batch 1 material, and closed symbols represent batch 2 material. Error bars represent one s.d. of the mean.

Plate 7.1

Results of a tetrazolium test on desiccated *Carica papaya* L. seed embryos, the deep red staining indicates high levels of seed viability, moisture content was 4.6 ± 0.4 %.

Seeds that failed to germinate at 26 °C after desiccation, were tested for viability using a standard TZ salt test, which gave positive results (see Plate 7.1). This indicated that desiccation must have induced dormancy. The 33 / 19 °C alternating temperature regime was, therefore, believed to have remove this dormancy effect. However, the benefits of the 33 / 19 °C treatment were 'short-lived' as the germination levels start to fall off after c. 2 to 4 d of drying. As a consequence of this, the beneficial effects of an alternative alternating temperature treatment upon papaya seed germination was further investigated. Pérez *et al.*, (1980) had some success germinating papaya seeds when they were exposed for very short time periods (15 seconds), to very high temperature (70 °C) water treatments, prior to sowing. To examine this response in more detail, a lower temperature was investigated, as this effectively extended the time of exposure, enabling the effects of both temperature and exposure time to be more clearly resolved.

Exposures to 36 °C during a 26 °C germination treatment was utilised as this represented a similar temperature amplitude fluctuation as the 33 / 19 °C treatment, which had been partially successful in removing dormancy. Initially the exposure to 36 °C lasted 7 d, but as the exposure time was reduced, germination was seen to increase, reaching a maximum (c. 80 %) after c. 4 h (Fig 7.3). If the exposure time was further reduced, germination levels fell to those of the controls (compare the extreme left of Fig 7.3 to the extreme right of Fig 7.2). Line fits revealed that seeds were more sensitive to decreasing exposure time ($y = 118.974 - 22.774x$, $r^2 = 0.68$), than increasing ($y = 28.448 - 4.215x$, $r^2 = 0.75$). Glim analysis (Crawley, 1993), using the GLIM 4 statistical package revealed that this response was not dependent upon light ($P < 0.05$), and hence, was not a phytochrome related response. Only treatments in the light were used hereafter.

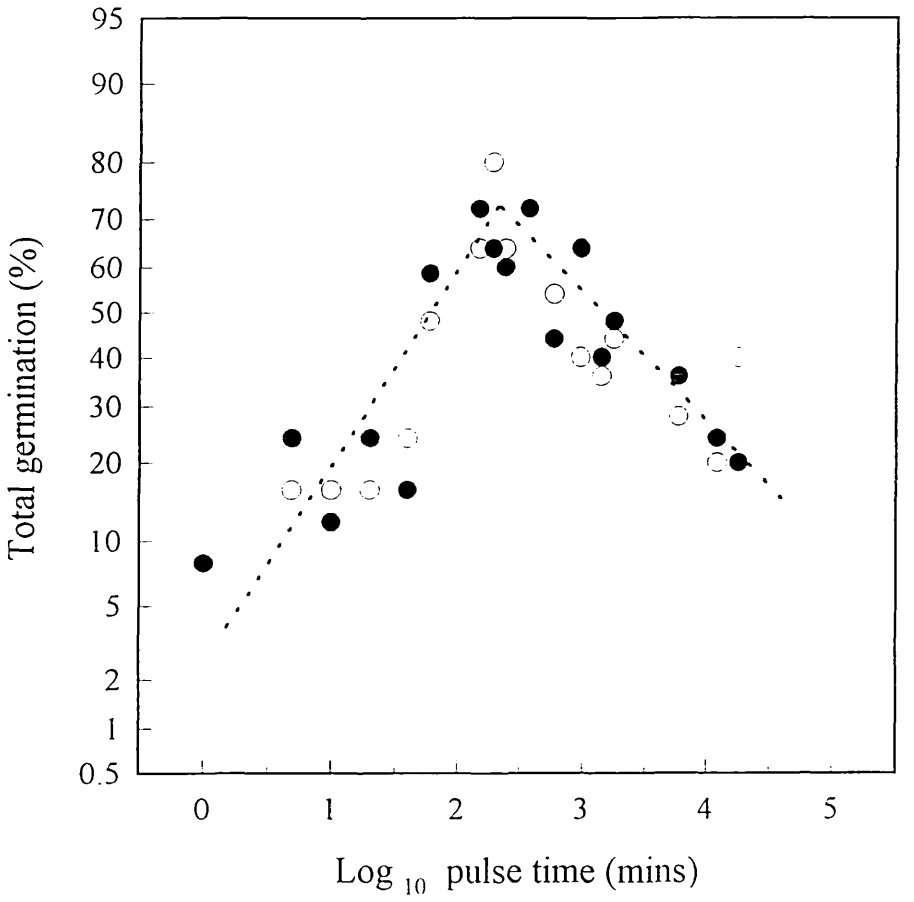


Figure 7.3 The effect of exposure time at 36 °C, during a 26 °C incubation period on the final germination percentage of *Carica papaya* L. seeds. The results are an average of batch 1 and 2 seeds, open symbols represent treatments performed in the light, closed symbols represent treatments performed in the dark. The fitted dotted line represent linear probit regressions, the line equations of which are (y = 118.974 - 22.774x, r^2 =0.68) and (y = 28.448 - 4.215x, r^2 =0.75) for the left and right hand line respectively.

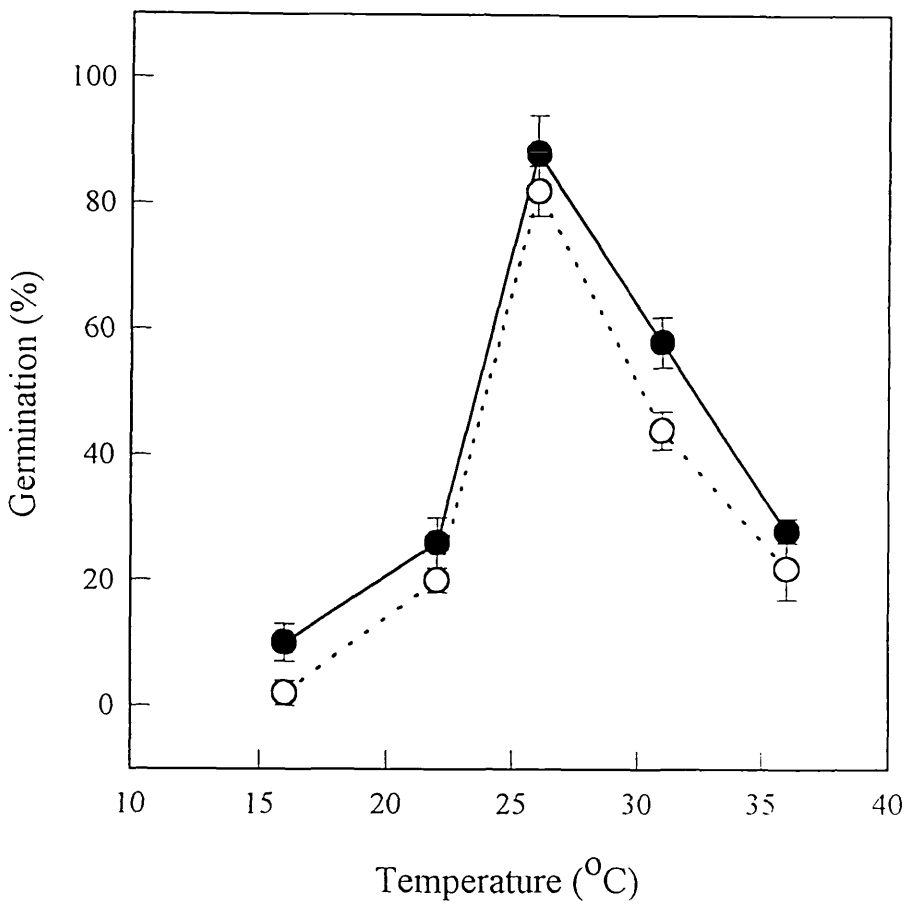


Figure 7.4

Relationship between incubation temperature both before (closed symbol) and after (open symbol) exposure to 36 °C and the average germination of batch 1 and 2 *Carica papaya* L. seeds. Error bars represent one s.d. of the mean.

The most effective incubation temperature both before and, after exposure to 36°C (for 4 h) to maximise germination was 26 °C (Fig 7.4). The use of temperatures either 10 °C higher or lower than the optimum, resulted in low germination (c. 20 %).

The time at which the 36 °C high temperature 'pulse' was applied also affected germinability. Papaya seeds were fully rehydrated after 24 h on agar (Fig 7.5). However, the ability to respond to temperature 'pulsing' was not evident 24 h later, only after 5 d, or more of rehydration did seeds germinate maximally. The fact that the embryo needs to reach > 25 % moisture content to respond to germination indicates that the seed requires some time to biochemically prime itself. This dependency on metabolism raises the question as to whether respiration was also affected during the 36 °C exposure.

Respiratory measurements conducted using naked batch 1 and 2 seeds (mesotesta removed) showed that during the 4 h exposure to 36 °C, within a 26 °C incubation period oxygen was consumed at a rate of 2400 ml g^{-1} d. wt. h^{-1} (Table 7.2), demonstrating a high metabolic dependency on oxygen. Oxygen availability was further demonstrated to be essential for the germination response, as seeds did not germinate in anoxic environments (either CO_2 or N_2). Hypoxia (100 % O_2) also reduced germination from c. 82 to 58 %. An increase in germination on subsequent transfer from oxygen to air was also observed, highlighting the requirement of specific oxygen concentrations.

7.3.2 Effects of chilling

Drying batch 1 and 2 papaya seeds for 1 and 4 d reduced germination at 26 °C to c. 20 %. A subsequent 1 d treatment at cool / cold temperatures down to -196 °C did not affect germination greatly (Fig 7.6). However, there were some anomalies in this

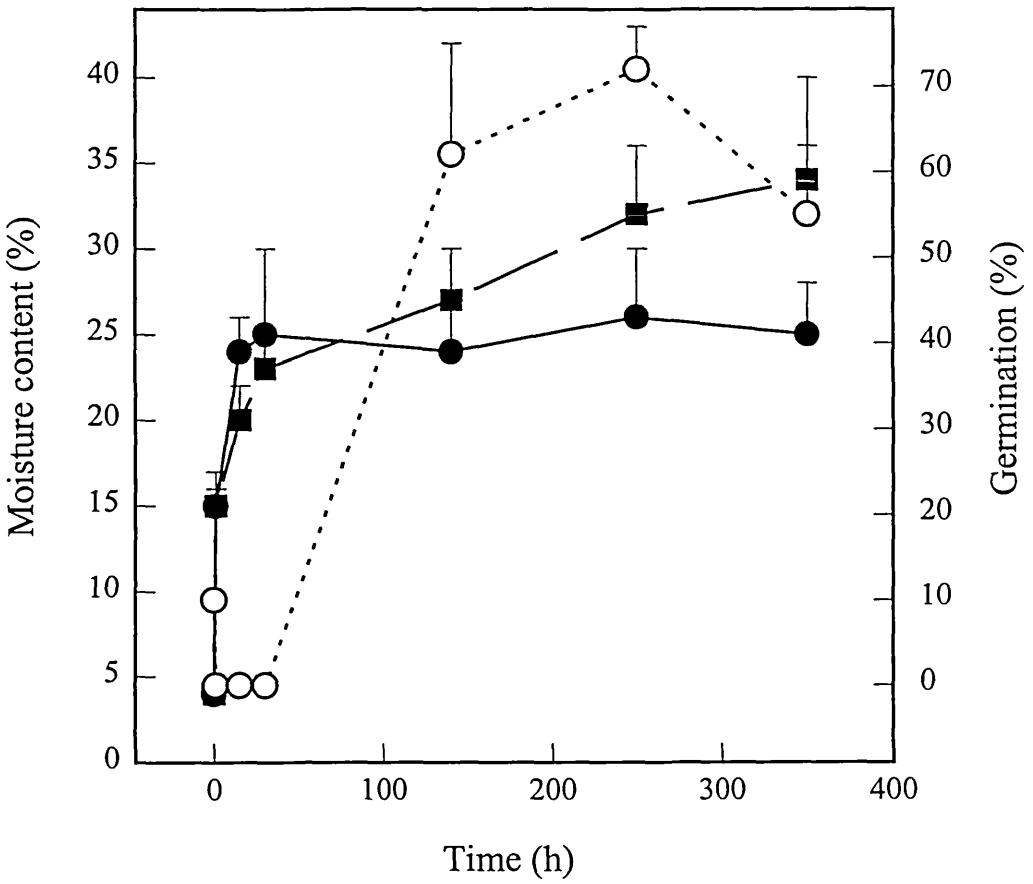


Figure 7.5

Relationship between moisture content (solid and dashed lines), germination (dotted line) and rehydration period of *Carica papaya* L. seed embryos (circles) and endosperm (squares). Error bars represent one s.d. of the mean.

Table 7.2

Effects of the high temperature 'pulse' on the oxygen consumption of Carica papaya *L. seeds before, during and after dormancy breakage (n = 3 x 5).*

Temperature treatment	Oxygen consumption (ml g^{-1} d. wt. h^{-1})
26 °C pre-pulse	800
36 °C 4 h 'pulse'	2,400
26 °C post-pulse	980

Table 7.3

Effect of storage time and temperature on the average germination of batch 1 and 2 Carica papaya *L. seeds using either 26 °C, 33 / 19 °C or 26 °C including a 36 °C 'pulse' for 4 h as germination treatments.*

	Storage temperature (°C)									
Germination temperature	-196	-70	-50	-30	-20	-13	2	6	11	16
	1 d Storage period									
26 °C	20 ± 2	22 ± 4	16 ± 2	18 ± 4	10 ± 2	0 ± 0	12 ± 4	26 ± 4	24 ± 4	14 ± 2
33 / 19 °C	92 ± 6	88 ± 2	86 ± 4	90 ± 2	80 ± 2	62 ± 6	92 ± 4	86 ± 2	92 ± 2	100 ± 0
36 °C pulse	80 ± 4	94 ± 2	92 ± 2	84 ± 4	84 ± 4	52 ± 3	92 ± 8	88 ± 0	86 ± 2	100 ± 0
	4 d Storage period									
26 °C	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
33 / 19 °C	52 ± 0	76 ± 4	92 ± 0	92 ± 8	69 ± 3	80 ± 4	64 ± 4	100 ± 0	94 ± 6	80 ± 4
36 °C pulse	56 ± 0	100 ± 0	84 ± 4	100 ± 0	80 ± 0	30 ± 2	94 ± 2	86 ± 6	98 ± 2	88 ± 0
	9 d Storage period									
26 °C	2 ± 2	0 ± 0	2 ± 2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2 ± 2
33 / 19 °C	49 ± 7	54 ± 2	68 ± 4	38 ± 2	54 ± 2	30 ± 2	68 ± 4	50 ± 2	72 ± 4	58 ± 2
36 °C pulse	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	40 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	13 d Storage period									
26 °C	0 ± 0	0 ± 0	2 ± 2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
33 / 19 °C	62 ± 2	76 ± 4	78 ± 2	68 ± 0	50 ± 6	18 ± 2	100 ± 0	100 ± 0	100 ± 0	100 ± 0
36 °C pulse	100 ± 0	98 ± 2	100 ± 0	100 ± 0	100 ± 0	36 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0

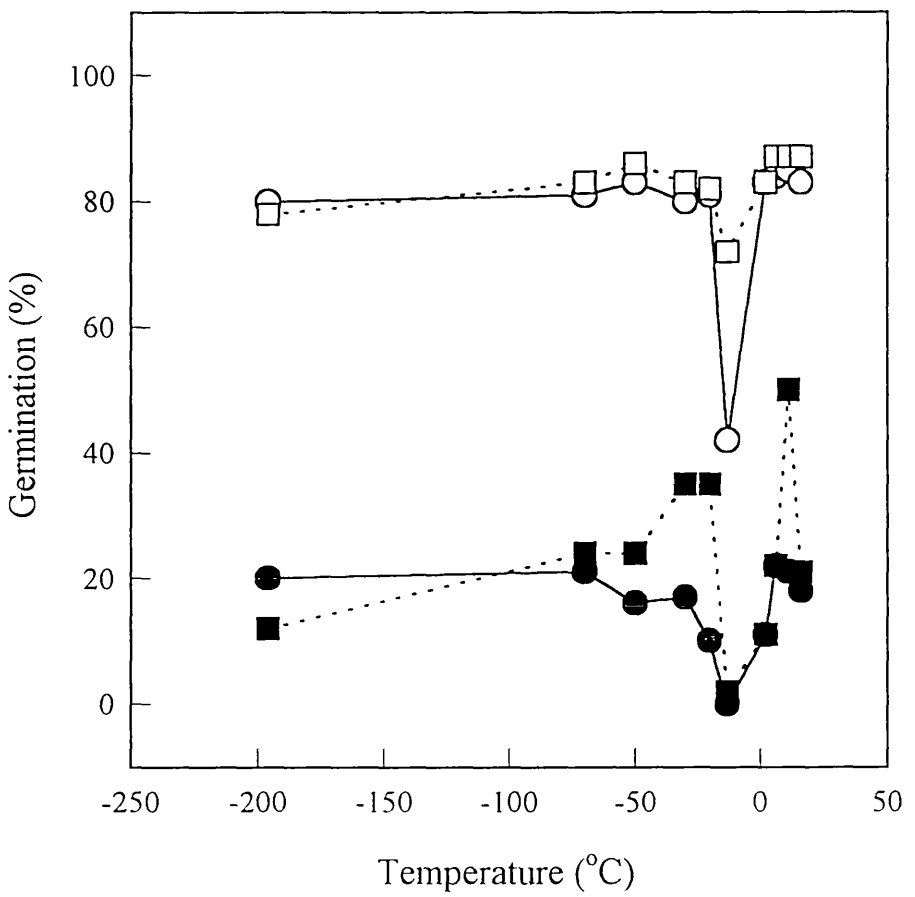


Figure 7.6

Relationship between storage temperature and germination for *Carica papaya* L. batch 1 (circles) and batch 2 (squares) seeds germinated at 26 °C (closed symbols) and at 26 °C using a 36 °C 4 h 'pulse' treatment (open symbols).

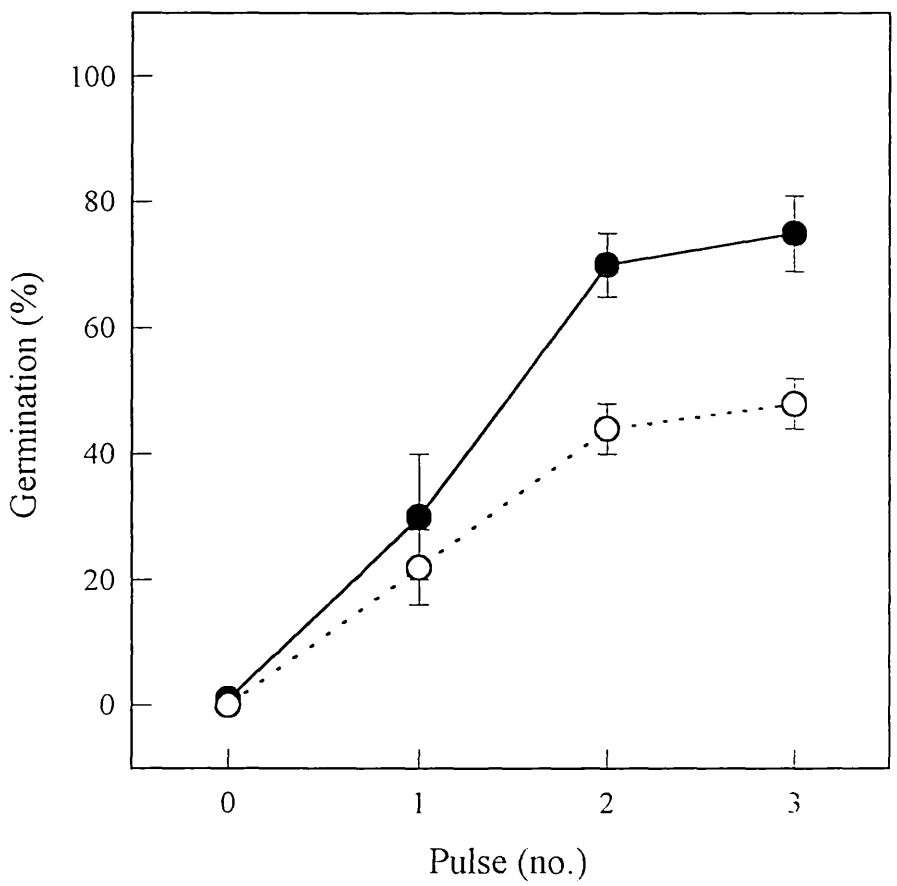


Figure 7.7

Relationship between the number of 4 h 'pulses' at 36 °C and germination of batch 3 *Carica papaya* L. seed stored for 1 d at either -13 °C (open symbols) or -20 °C (closed symbols). Error bars represent one s.d. of the mean.

relationship; seeds exposed to -13 °C gave lower germination values than the other storage temperatures. For example, germination at 26 °C and 26 °C including a 4 h 'pulse' at 36 °C was c. 0 and 70 % respectively (Fig 7.6; Table 7.3). A 33 / 19 °C germination temperature was also favourable if freezing at -13 °C was limited to between 1 to 4 d (Table 7.3). Longer periods of chilling / freezing generally resulted in lower germination levels at both 26 °C and 33 / 19 °C (Table 7.3). However the 4 h, 36 °C 'pulse' treatment continually gave enhanced levels of germination for all treatment including that at -13 °C (Table 7.3).

The results suggested that temperatures close to those used in conventional seeds banks induced dormancy. To determine if this was the case, seeds dried for 7 d were frozen for 1 d at -20 and -13 °C. In this experiment, a different seed lot (batch 3) was used to those in previous experiments (batch 1 and 2), as more seeds were required. However, a single exposure of the seed to 36 °C after 14 d at 26 °C raised germination to between c. 25 - 27 %, initial germination after treatment was ≤ 2 % (Fig 7.7). Two additional pulses further increased germination to c. 49 - 77 %, for seed previously frozen at -13 and -20 °C (Fig 7.7).

7.4 Discussion

Current seed banking methodology relies upon the fact that dehydration and cold storage of seeds extends the storage period during which their viability can be retained. Many seeds can tolerate desiccation to 1-5 % moisture content, and storage at temperatures down to -20 °C; such seeds are termed 'orthodox' (section 1.1.2). Harrington (1972) suggests that in orthodox species, seed longevity can be doubled for

every 1 % decrease in moisture content, or 5 °C decrease in temperature (between the ranges 14-5 % moisture content and 50-0 °C).

For many years papaya seed had been described as desiccation intolerant (recalcitrant; Teng and Hor, 1976) but more recently Ellis *et al.*, (1991b) have suggested that the storage characteristics of this seed are more suited to the 'intermediate' classification (discussed by Ellis *et al.*, 1989 and 1990 for coffee and papaya) as the germination characteristics fell between those of orthodox (see above) and recalcitrant (see section 1.1.1.2). There has been some dispute surrounding this categorisation as it has also been noted that some orthodox seeds also display sensitivity to freezing (Oilpalm, Grout *et al.*, 1983; *Araucaria*, Tompsett, 1983; sunflower, Vetucci, 1989; orchid seed, Seaton and Hailes, 1989, Pritchard and Seaton, 1993; *Agathis*, Dickie and Smith, 1995; Pritchard also reviewed this subject generally in 1995). Papaya seeds have been found to be notoriously difficult to germinate (Lange, 1961, Ellis *et al.*, 1991b and Magill, 1993), but can survive desiccation and freezing within narrowly defined limits. However, not within the limits generally associated with orthodox seeds. Unfortunately, viability assessment post-treatment generally involves germination and it is known that the sensitivity of seeds to external stimuli (temperature, moisture ect.) can alter *after* drying.

For instance, even dry orthodox seeds when exposed to excess free water, can suffer from imbibitional stresses caused by a rapid influx of water to sensitive seed tissues such as the embryonic axis. This may result in problems such as efflux of solutes, an increased chance of infection, and deleterious membrane phase alterations (Hoekstra, 1989; Ellis *et al.*, 1990). Moreover, such responses could be construed as reflecting and 'intermediate' type behaviour.

To avoid these problems when studying the germination characteristics of dried papaya seeds, direct rehydration took place on the 1 % agar water used in for routine germination tests. The alternative method of hydrating first *over* water (at 21 °C) was also investigated, and did not affect germination levels. As agar water was to be used for germination studies this method of rehydration was adopted, as it eliminated the water rehydration step. Agar water rehydration also resulted in a more rapid imbibition rate, this seemed to give the seeds a slight 'head-start' in terms of germination, After a 25 d period germination was 12.5 % higher in seeds rehydrated on 1 % agar water, compared to those rehydrated over distilled water (data not shown).

It was also useful to determine the variation in 'physical index' of fruits and seeds (from both between and within fruits). The finding that seeds and fruits displayed considerable similarity (Table 7.1) suggested a low variation in developmental ages, and enabled the seeds from different fruits to be 'pooled' and treated as a single seedlot. This was important as Ellis *et al.*, (1991a) had suggested that possible differences in the stages of development of coffee seed was responsible for a variation in the intermediate response between seed lots.

7.4.1 Effect of temperature on germination

Magill (1993) had some success germinating papaya seeds at incubation temperatures of both 26 and 36°C, with the lower temperature giving the best levels of germination, and Ellis *et al.*, (1991b) had some success using a 20 / 30 °C alternating temperature regime. A further investigation of these temperatures was therefore an obvious place to start, and the results from these experiments were discussed earlier.

The subsequent germination studies of papaya seed using a high temperature 'pulsing' technique (see earlier sections of this chapter) revealed that although the species has been described as 'intermediate' (Ellis *et al.*, 1991), this classification may be due to the initiation of dormancy within this species upon drying. This appears to be effectively broken by short exposures to an elevated temperature, even when the seed has been previously dried to very low moisture contents (beyond that of both typical intermediate and recalcitrant seed survival).

From my germination results it can be seen that papaya seeds can be dried without loss of viability, however, their dormancy status is enhanced. This can be overcome efficiently through the short term application of a single pulse of high temperature. Although many different features of the temperature control of dormancy loss in seeds have been identified, including amplitude, thermoperiod and temperature optima (Murdoch *et al.*, 1989), it is usual that diurnal temperature alterations are required over a few weeks, for example in *Chenopodium album* L. (Murdoch *et al.*, 1989). By comparison, the temperature sensitivity of papaya seed is finely tuned, a single 10 °C alteration in temperature being sufficient to elicit the response

When the results of the high temperature alteration treatments are plotted, a normal distribution around both the optimal germination temperature and incubation period can be seen. Probit analysis of the effect of the 36°C exposure time period on germination confirmed that approximately 4 h was optimal for dormancy breakage (Fig 7.3) Temperatures of 16, 21, 26 and 31°C for the 'pulse' were not effective; typical germination levels using these temperatures were 0-10% after 35 d in the germination tests (data not

shown). No statistical difference ($P < 0.05$), was observed in the sensitivity of the seeds to increasing time at high temperature in response to the presence or absence of light. If the 'pre-pulse' conditions employed light when the 'post-pulse' procedure was performed in the dark (or *vice versa*), germination was reduced by 35 % to 45%. Seeds maintained in the dark during the whole treatment, or only receiving light during the pulse period, had germination levels of approximately 10%. So although light is not involved in the release of dormancy, it is clearly needed for germination to progress satisfactorily.

7.4.2 The effect of other external factors on papaya seed germination

Dried papaya seeds quickly re-hydrate upon agar, or over water, and when moist, can germinate successfully in the right conditions. But because of the slow germination observed at constant temperatures (26 and 36 °C), it appears that papaya seeds may be deeply dormant. The elevated percentage germination rates achieved using alternating temperatures appear to demonstrate that this technique may be releasing the seeds from dormancy. However, although it appeared that temperature alteration can be the major dormancy breaking mechanism, it was still not clear what effect other parameters had on influencing the breakage of dormancy.

Several external factors can determine the progression and rate of seed germination, one of which is light. For many seeds germination is initiated by light through the interaction of phytochrome (see Bewley and Black, 1982 for a review). The observation that the high temperature 'pulse' response was not light sensitive (Fig 7.3), suggests that there was no major interaction between high temperature and phytochrome in papaya seeds.

Oxygen concentrations can also determine both the expression, and progression of, germination. The results of germination in varying oxygen concentrations revealed that oxygen was a requirement for germination in papaya, however, too high a concentration of oxygen was also detrimental. This indicates that germinating papaya seeds need to process oxygen at specific concentrations in order to germinate.

Previous studies have shown the survival of desiccation by papaya seeds (Chapter 5; Magill *et al.*, 1994), this suggests that papaya seeds may have a metabolism that can efficiently process activated oxygen products that may accumulate from respiratory metabolism during desiccation stress. This is in direct contrast to the biochemical response of previously investigated recalcitrant material which cannot respond in this way. Desiccation sensitive material appears to suffer from an inability to process products of a highly active oxygen metabolism, and as a consequence viability is reduced upon severe desiccation (see Chapters 4, 5 and 6).

In summary, the loss of seed dormancy in papaya appears to be controlled by temperature and the gaseous environment, with the first parameter being effective only when oxygen levels are optimal. It may be that this temperature response is related to the production of a defined set of proteins called heat shock proteins (HSPs) (Parsell and Lindquist, 1993, Nover, 1991, Vierling, 1991 and Morimoto *et* al., 1994). These 'molecular chaperones' are ubiquitous in nature, but usually abundant and diverse in higher plants. It is likely that one of the functions of HSPs in plants is related to specific developmental processes (Waters *et* al., 1996), one of which may, perhaps, be dormancy release.

The importance of oxygen for seed germination in this species has been previously observed in relation to the use of aerated water as a pre-germination treatment (Pérez *et al.*, 1980). In addition to oxygen, light appears to play an important role in both pre- and post-pulse temperature treatments. As contrasting effects of light on respiration in seeds have been reported (Vertucci and Leopold, 1987), it is possible that light has a direct effect on the oxidative status and the germinability of the seeds.

7.4.3 Chilling sensitivity

Determinations were made on the cold temperature sensitivity response of papaya seed germination, to compare with the effects of desiccation previously investigated. For orthodox species the two parameters of temperature and seed moisture content are closely related (section 1.1.2). There have also been reports that have shown, that other putative-intermediate seeds such as neem can actually survive freezing (Berjack *et al.*, 1995), and indeed Becwar *et al.*, (1983) has already demonstrated that papaya can survive a 1 d liquid nitrogen storage treatment. It was the purpose of this section of the study to investigate papaya seed germination, and to try an reconcile the contradictions observed when freezing sensitivity of intermediate seeds is described.

In this regard, the investigations of chilling and freezing of desiccated papaya seed, showed that such treatments enhanced the level of seed dormancy, particularly at certain critical temperatures (c. -13 °C). The results indicate therefore, that the breakage of dormancy by alternating temperature effect is affected by storage temperature reduction, although this is only really seen when desiccation is combined with freezing in a certain narrow range of temperatures close to those used in conventional seed banks. The sensitivity of papaya seed

to -13 °C, even using the 'pulsing' technique indicates that this temperature may not be conducive for the long term storage of such material.

To conclude, it appears therefore, that the storage classification of papaya seed as 'intermediate' may be incorrect. It's germination previously being confounded by desiccation and chilling induced dormancy, which may have contributed to the seeds possible misclassification.

There are two implications to be drawn from this hypothesis:

1. The response to chilling is particularly important as the sensitivity to temperatures around freezing on dormancy induction, may have consequences for storing this type of material at conventional seed bank temperatures. If one is not sure of the germination protocol that may alleviate the imposed dormancy response, the conventional storage of such material becomes questionable. This is particularly relevant as we are not sure if other 'intermediate' seeds (e.g. coffee) also respond in this way. The storage of such seed at alternative (sub-zero) may therefore need consideration.

2. Perhaps more importantly is the fact that the response itself casts some doubt on the accepted intermediate classification (see Berjak and Pammenter, 1994). Papaya seed can obviously survive drying well below that described for its storage classification, and indeed its miss-classification may be a result of its desiccation / freezing induced dormancy. In support of this is the finding that a similar survival of extreme drying

has also been demonstrated within a population of another 'intermediate' seed, i.e. neem (see Chapter 5).

7.5 Summary

Fresh papaya seeds germinated at constant temperatures in the light (12 h), following the trend 26 > 36 °C. The ability to germinate at these temperatures was lost however, when fresh seeds were dried to < 10 % moisture content. In contrast, the ability to germinate at an alternating temperature (33 / 19 °C) was not lost. The effect of drying was not immediate, as germination at 26 °C decreased from day 1 onwards, but moisture contents between day 1 and 9 did not significantly alter. Also, seeds which failed to germinate at 26 °C, germinated when transferred to 33 / 19 °C.

The data demonstrates that desiccation within the zone of water sorption coincidental with the intermediate classification, may lead to an altered level of dormancy. This raises the question of whether dormancy contributes to the apparent intermediate effect (see Roberts *et al.*, 1984 for Citrus and Oil palm). To investigate this germination was looked at further, and although a constant temperature of 36 °C resulted in low germination levels, an optimum exposure time could be determined. As exposure times to 36 °C was reduced to below 7 d, germination increased, reaching a maximum at approximately 4 h. This response was not dependent upon light, and 26 °C was found to be the best incubation temperature for 36 °C exposures. The time of application of the 36 °C exposure during rehydration was also important, the seeds did not respond unless they had 'equilibrated' to an appropriately high moisture content.

The introduction of a 1 d chilling or freezing period after desiccation, did not systematically effect germination. However, seeds exposed to -13 °C gave lower values that the other temperatures. Longer chilling / freezing periods were detrimental, but the effects of all such treatments could again be alleviated by short term exposures to 36 °C. These results have implications for the accepted categorisation of such seeds as 'intermediate'.

CHAPTER 8

General Discussion

Chapter 8: General Discussion

The role of oxidative stress in seed survival was first discussed nearly three decades ago by Kookstra and Harrington (1969), and although there have been many studies in the intervening period, the subject has received relatively little detailed attention. This is most probably due to the dynamic nature of the systems under investigation, combined with the fact that so many previous reports have produced conflicting results (section 1.2.1.3).

Some these problems may be attributed to the difficulties one has to address when studying the systems involved in oxidative stress. Firstly, the biochemical and biophysical components of such systems are invariably complex, and secondly they can be extremely transient. Therefore, before a meaningful investigation of these systems can begin two major questions needs addressing: (i) which parts of the biochemistry / biophysics should be studied; and can the results gleaned from such disparate measurements be meaningfully related to each other, and (ii) how to accurately measure the events associated with the biochemistry and biophysics of such systems.

The first of these questions is relatively easily answered when regarding seed studies. Of the many investigations into the seeds biology, primarily the focus has been directed towards seed deterioration, and especially in the involvement of water loss in recalcitrant seed viability loss. As far as seed survival is concerned, the main question relating to viability loss is "Why do some seeds survive drying, whilst others do not ?",

this is not necessarily a question relating to any eco-physiological aspects of the seed, but has more recently been aimed towards the underlying causal mechanisms of viability loss.

The second question is, however, more difficult to answer, as there are many ways of measuring oxidative stress in biological systems (section 1.2.3). A balance is needed between the measurement of the precursors of oxidative stress (i.e. free radicals) and the quantifiable intermediates and products, so that a sequence of events can be proposed. Moreover, the examination needs to be carried out in detail as the results obtained from either aspect can influence an overall interpretation of biological reactions taking place within the seed, and hence, the physiological response.

Dealing with the latter of these two points first, an examination of free radicals within seed material using EPR spectroscopy (sections 3.3.1 to 3.4.3) revealed that several fundamental factors need to be considered before the technique can be widely, and comparably, applied to seed desiccation intolerance studies. The most important of which being the effects of type III-IV (unbound) water on EPR signal intensity. Of the many such studies previously described, the majority have conducted EPR measurements at ambient temperatures (sections 1.2.1.2 and 1.2.1.3). However, the temperature of measurement profoundly affects the interpretation of spectra. At ambient temperatures type III water 'quenches' the free radical signal intensity. Such effects have been known to spectroscopists for many years (Knowles *et al.*, 1976). However, the effects have been less appreciated by seed biologists / biochemists in recent studies of seed desiccation (Leprince *et al.*, 1990, Hendry *et al.*, 1992 and Finch-Savage *et al.*, 1993). Unfortunately, in many recalcitrant seeds maximum masking of the free radical

signal using ambient measurements coincides with moisture content regions where viability loss commences (i.e. c. 50 %).

To overcome this problem measurements in this study were made at 77 K (Chapters 3, 4, 5 and 6). When this was further combined with the use of second derivative EPR scanning, additional features of the free radical signal within seed material were observed. Specifically, in many species two free radical peaks were identified: a LF and HF signal. Drying of desiccation sensitive seed material in this study resulted in an increase in the LF signal, suggesting that more than one free radical species may be present within seeds. In contrast, other workers (Hendry *et al.*, 1992) using ambient measurement temperatures, have ascribed physiological significance to an increase in the HF signal upon desiccation. It has been postulated that both the LF and HF signals derive from a quinone free radical, which have been assigned similar g-values to those which I have reported (Atherton *et al.*, 1993). This assumption stems from the fact that the HF peak has not previously been associated with fully viable material, because its absolute intensity was infinitesimally small than in samples of high moisture content due to type III water quenching.

As the physical removal of type III water does not affect signal intensity when measured at sub-zero temperatures, the EPR signal intensity also more accurately reflects the level of radical activity. Previous reports (Hendry *et* al., 1992 and Finch-Savage *et al.*, 1994) have thus reported much larger increases upon desiccation of recalcitrant seeds, than I report; c. 100-fold for *Quercus rubra*, *Castania sativa* and *Aesculus hippocastnum*. As these measurements were conducted at room temperature, I believe the free radical

signal intensity was elevated upon water removal, and hence cannot be directly equated with free radical contents at high water contents (see Goodman *et al.*, 1995).

The precise identification of the free radical species involved in the response to desiccation of recalcitrant seeds is problematic due to the overlap of free radical signals, and the lack of hyperfine structure in the signal (mainly because of the intrinsic low signal : noise ratios obtained from biological material). Future work should be aimed at examining this aspect of EPR investigation to move the debate forward, through the use of more advanced EPR techniques such as Q and W-band spectroscopy, at appropriate measurement temperatures. This would increase the distance between the LF and HF free radical peaks, increasing their g-value resolution, and making identification of the free radical species more likely.

The main problem when studying free radicals is that they are extremely transient (i.e. some have half-lives of 10^{-9} seconds), I believe that the relatively 'stable' free radicals measured in this, and other investigations, may have been formed from more reactive, and hence less stable, precursors. Thus, a 'smoke trail' of destruction ensues, which never eludes to it's cause. In this regard, enhancements in free radical identification could be gained by using chemical 'spin traps'. These react with very short-lived radicals to form a more stable radical adduct, which can then be measured via EPR spectroscopy.

Additionally, although there was no evidence of a dramatic changes in width of the free radical peaks obtained in this study, theoretically the width of the peak could also have a contribution to the overall signal intensity. Further clarity of free radical accumulation

could therefore be gained if the area under the free radical peak was measured, instead of just the mm deflection. This is now more easily achieved with the introduction of specialised EPR spectral-analysis software such as 'Maxent' (Bruker Spectrospin, Coventry, U.K.), which can accurately calculate the double-integral data needed to assess peak areas (Goodman and Deighton, pers. com.).

The physiological quality of the seed tissue and its pigmentation also affected the free radical signal intensity. Oak seed embryos of low quality and suffering from necrosis had elevated free radical intensities, which indicates how important it is to carefully evaluate the health of material before EPR measurement. It has also been seen that consideration needs to be given to seed pigmentation, through the observation that pigmented neem seed mesotestas gave very high free radical signal intensities. The observation that free radical accumulation can occur to a greater extent in pigmented seeds coats than in other seed tissues has also been reported by Khan *et al.*, (1996) for soybean testas. In this study the question was raised as to whether the testa may offer some protective function. It also furthered the debate as to the inherent differences that may occur in oxidative responses between tissues, due to their contrasting organic composition. For this reason it is useful to study, where possible, isolated parts of the seed, as some give more free radical activity than others. This seems dependent mainly upon their metabolic activity, pigmentation and tissue quality (section 3.4.1 to 3.4.3). The study of isolated tissue also gives enhanced sensitivity to biochemical investigations, as the effects of bulk changes that may occur in neighbouring tissues are eliminated.

Studies of tropical desiccation intolerant material revealed that differences exist in the intensity and nature of the oxidative response to desiccation between species. In general, the free radical response is similar to that of horse chestnut, signals comprising of a LF and a HF signal, the LF signal increasing upon viability loss. However, neem seed displayed only a LF signal, which also increased upon desiccation. This may be related to the fact that neem seeds lose viability in a different water sorption zone (Fig 8.1) to that of the other desiccation sensitive seeds studied. Moreover, the fact that products of lipid peroxidation accumulate in neem seeds in the type II water sorption zone may indicate that different, non-enzymic, oxidative process occur in this species, compared to seeds with higher desiccation sensitivities (St. Angelo, 1992). Future studies could establish if the intermediate response to drying is only ever accompanied by an increase in the LF signal intensity, this would address the issue of whether there is a characteristic feature of the intermediate response to desiccation.

It is however, possible that the difference in oxidative response of neem may, be related to damage during imbibition prior to germination, rather than the effects of desiccation *per se*. For this reason it would be useful to study the oxidative stress response at different stages during rehydration prior to germination. For example, Leprince *et* al., (1998) found that neem seed endosperm contained relatively small amounts of oleosins, interfacial proteins which surround and stabilise oil bodies. It is thought that the shortage of these proteins allow oil bodies to coalesce during the imbibition of dry neem seeds, resulting in a reduction in the effective area of the oil body available for reserve metabolism and hence a loss of cellular integrity. This may contribute toward a loss of viability during the imbibition process involved in the germination of neem seeds following desiccation. It is clear, therefore, that to gain an overall picture of cellular

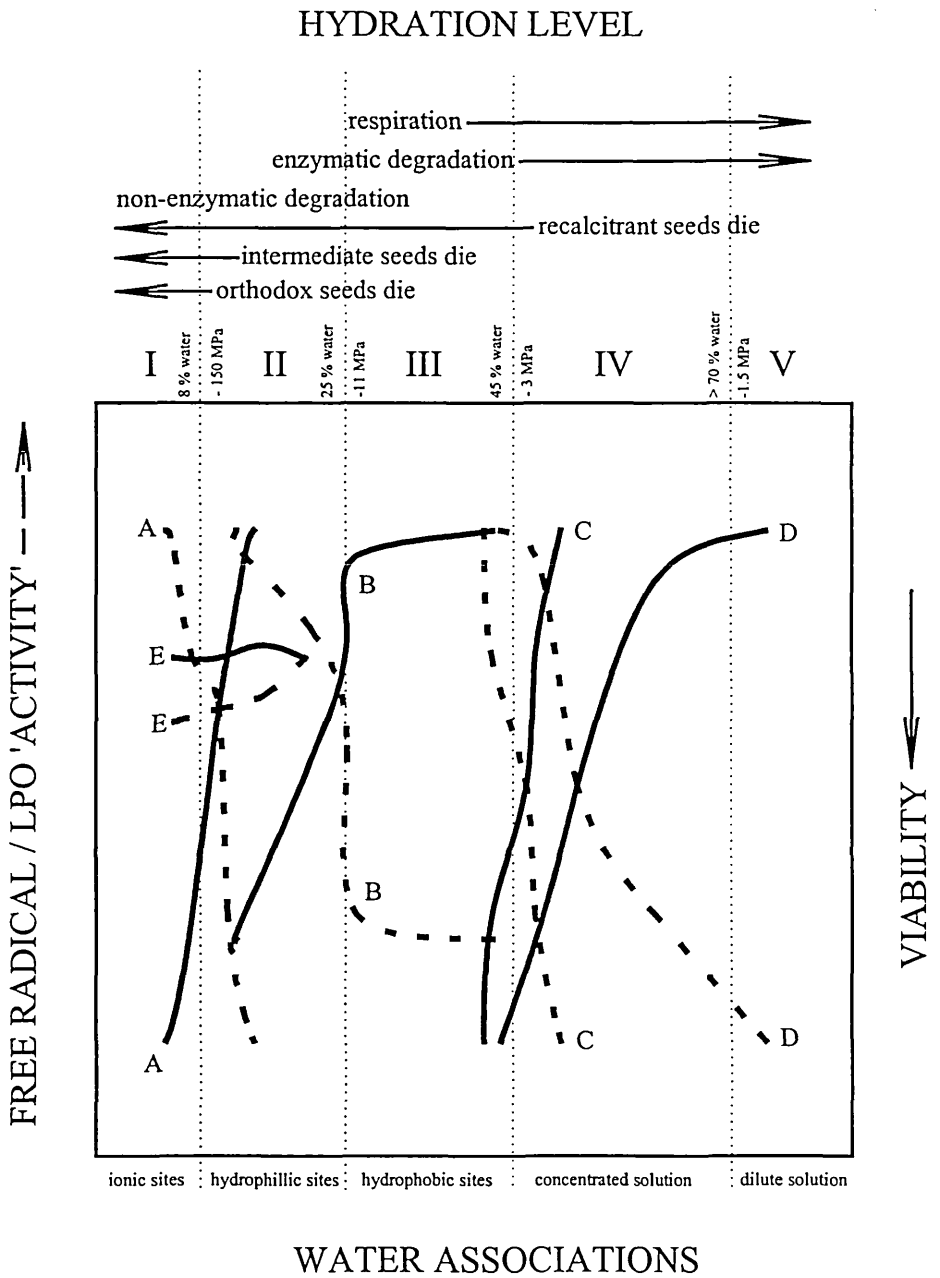


Figure 8.1 Schematic representation of the relationship between hydration level, viability (solid line) and the free radical / lipid peroxidation (dashed line) response of (A) *Azadirachta indica* L., (B) *Aesculus hippocastanum* L., (C) *Auracaria angustifolia* L., (D) *Theobroma cacao* L. and (E) *Carica papaya* L. seed components (which are mainly embryonic, see relevant chapters for detail). Critical moisture levels are given, as are approximate water potentials. The realtionship between these two measurements of water behaviour are also related to key events in seed viability loss (portions adapted *after* Vertucci and Farrant, 1995).

activity the results of seemingly unrelated biophysical and biochemical investigations into seed survival need to be considered together and not in isolation.

Species studied displayed varying desiccation intolerances, however, they have similar oxidative responses. However the fact that this occurs over a wide range of moisture contents indicates that there may be more than one free radical precursor responsible for intrinsically similar biochemical responses to desiccation. This finding is supported by the variation in g-values of free radical peaks found between species (Table 8.1).

Table 8.1
Variation in g-value of free radical peaks observed between the species studied, in order of increasing desiccation sensitivity.

Species	LF g-value	HF g-value
Carica papaya	**2.0041 ± 0.010**	**2.0008 ± 0.005**
Azadirachta indica	**2.0150 ± 0.050**	-
Aesculus hippocastanum	**2.0060 ± 0.015**	**2.0020 ± 0.005**
Quercus rubra	**2.0040 ± 0.005**	**2.0015 ± 0.015**
Quercus robur	**2.0051 ± 0.005**	**2.0026 ± 0.001**
Aricaria angustifolia	**2.0016 ± 0.010**	**2.0001 ± 0.005**
Theobroma cacao	**2.0113 ± 0.020**	**2.0087 ± 0.010**

Associated with the increase in free radical accumulation was an increase in the products of lipid peroxidation, which were easily detected in viable seed material via aqueous extraction and an established fluorimetric determination of TBARS (Fraga *et* al., 1988). Moreover, the use of this technique enabled me to evaluate assay procedures that had not been previously applied to seed based systems, *viz* the LPO-586 kit for the determination of 4-HNE and MDA, and the LPO-K (CC) assay which quantified total

lipid peroxidation concentration. The validation of these kits has extended their use beyond medical diagnosis (see Chapter 4 for discussion). This is the first example of such specific assay techniques being applied to seed biology, and has resulted in the detection of 4-HNE for the first time in seed material. These medically derived kits proved to be more sensitive than traditionally used assay techniques (smaller s.d. errors), however, they are more expensive, and this may limit their use some what. The detection an identification of lipid peroxidation products and aldehydics is obviously beneficial in studying the chemistry of seed ageing / senescence, and future work should therefore be aimed at further characterising the chemicals involved in oxidative stress. Perhaps through the use of more advanced analytical techniques such as HPLC and LC-MS (e.g. Deighton *et al.*, 1997). Moreover, the movement of water in desiccating seeds and / or seed components may be visualised by modern NMR-imaging techniques (Pritchard and Glidewell, pers. com.), and this may enhance the interpretation of differential desiccation sensitivity of various seed components.

Other biochemical assays used in this study measured various antioxidants in desiccating and stored horse chestnut seeds and revealed little activity. It is presumably a combination of the enhanced oxidative stress response, and the inefficiency of the antioxidants studied which contributed to viability loss upon drying. However, in this study only water soluble antioxidant systems were studied (due to the constraints of time and seed numbers), it would be interesting to expand this area of investigation to include other antioxidant mechanisms, which are not necessarily water soluble. The most important of which would possibly be the glutathione recycling system, concentrations of vitamins A and E in addition to a detailed study of the ascorbate peroxidase system. This would give a clearer view of antioxidant activity within the

seed, and may help to answer why recalcitrant seeds suffer from oxidative activity upon stress related to desiccation and storage, and why desiccation tolerant seeds do not.

The finding that papaya seeds survive desiccation, and do not accumulate free radicals gives further support to the hypothesis that oxidative stress responses are involved in the loss of viability that is exhibited upon drying of desiccation-sensitive seeds. This increase in oxidative activity when desiccation sensitive material is taken below a critical moisture content may serve as a useful diagnostic marker in determining seed storage behaviour. Tropical seeds tend to have higher desiccation sensitivities than temperate material e.g. *Theobroma cacao* (Chapter 5), *Avicenia marina* (Berkjak *et al.*, 1984) and *Inga* (Pritchard *et al.*, 1995a), because of this future work should concentrate on extending these studies to cover a wider range of species in addition to those discussed in this thesis, to further elucidate these complex mechanisms and their possible diagnostic value. An outline of the possible mechanism of the oxidative repsonse to desiccation can be found in section 4.4.3.

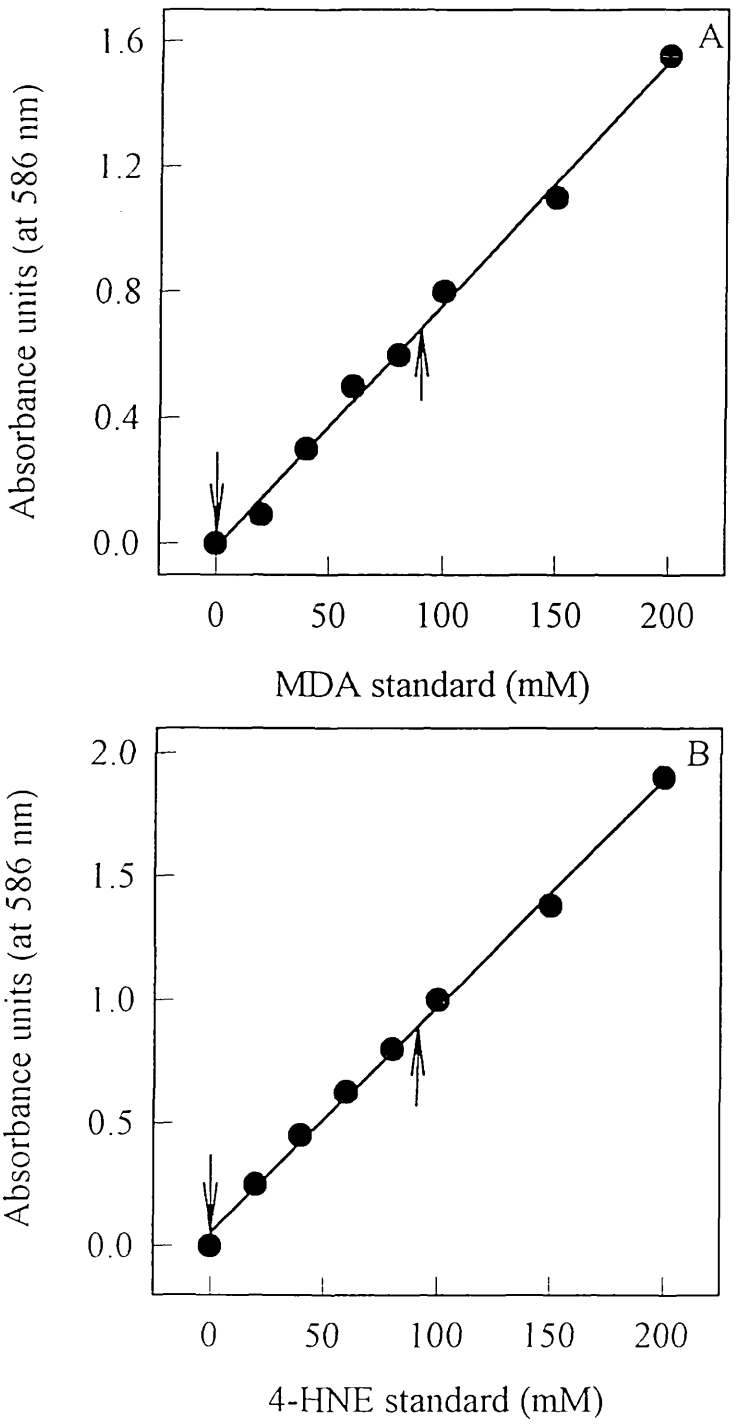
Lipid peroxidation and free radical accumulation increases seen during the hydrated storage of recalcitrant material, is presumably linked to the resultant increase in desiccation sensitivity also seen upon storage. It seems therefore that oxygen metabolism has a modulating effect on cell deterioration during storage. In contrast, the loss of viability observed when a desiccation tolerant species is stored dry, appears to have no connection to oxidative metabolism.

It is also interesting to note that in papaya seed at least, desiccation does have an effect on the seed, but one that is not related to viability loss. This is the induction of

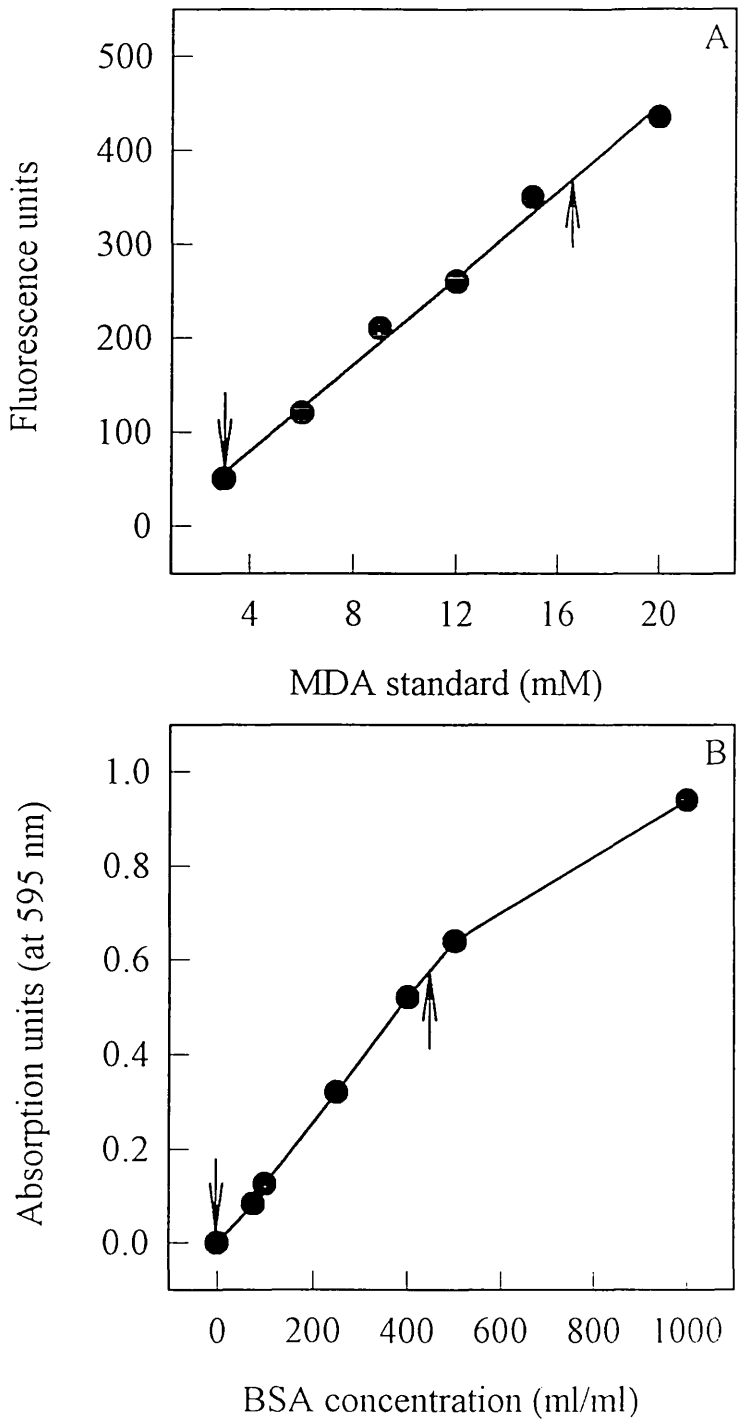
dormancy when seed is dried and / or frozen, which has consequences for both the current storage categorisation of this species, and its storage environment. The findings that papaya seeds survive desiccation, and do not show an increase in oxidative stress products gives credence to its re-classification as an orthodox seed. In support of this are the findings of Steadman *et al.*, (1996), who studied the ratios of tissue soluble specific sugars within different seed species, covering a range of storage categories. A correlation was drawn between sugar ratios and seed storage category. There were however one or two anomalies, one of which was the finding that embryos and endosperms of papaya seeds contained sugar ratios that were more akin to those found for orthodox seeds. Indeed Magill *et al.*, (1994) have also show that this species can be dried to very low moisture contents, without a reduction in seed viability. It may be that this could be one of many cases of intermediate seed storage type misclassification (e.g. Roberts and King, 1980 and Kovach and Bradford, 1992).

Finally, investigations into seed oxidative responses should continue, at a more cellular level, looking in detail at specific parts of the biochemical and physical response. Moreover, the fact that many oxidative products react perniciously with proteins, or the thiol groups of proteins, indicates an area of study that needs urgent attention. The relationship between oxidative stress responses associated with viability loss and genetic stability has been seriously overlooked. An investigation of this type is extremely important as seeds are maintained for both agronomic and conservation purposes, where the maintenance of genetic stability is the primary goal (Boubriak *et al.*, 1997).

Appendix I

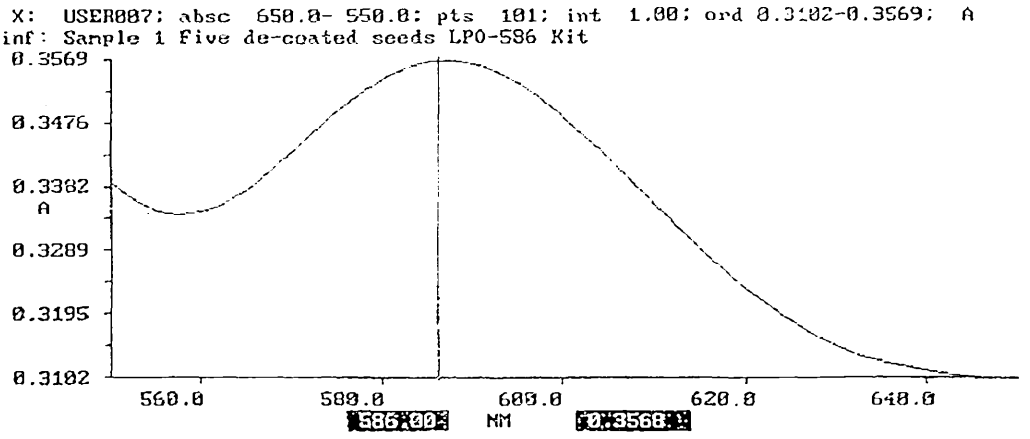


Example MDA (A) and 4-HNE (B) standard curves derived using the Bioxytech LPO-586 assay kit. The arrows represent the concentration range within which seed extractions fell, the fitted lines are first order regressions, $r^2 = 0.99$ for both (A) and (B).

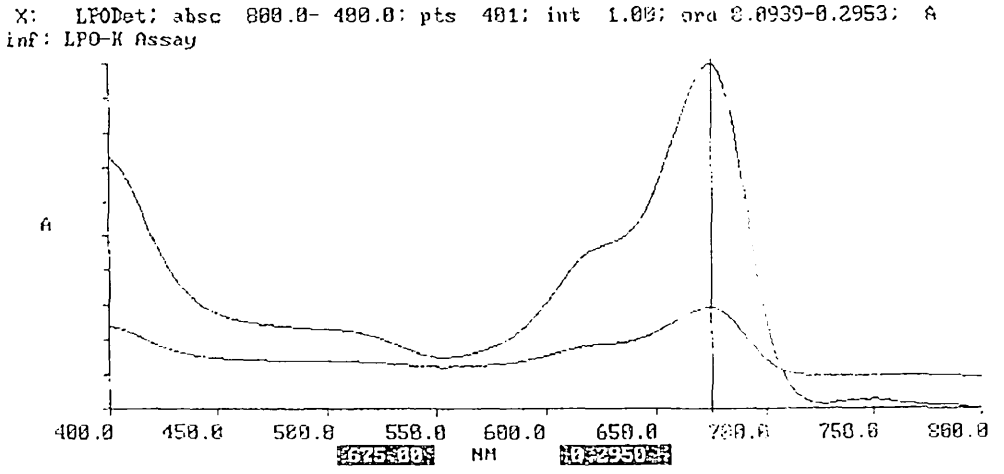


Example fluorescence (A) and BSA (B) standard curves derived using the TBARS (measured as MDA equivalents) and Coomassie assays respectively. The arrows represent the concentration range within which seed extractions fell, the fitted lines are first order regressions, r^2 = 0.99 for (A) and 0.96 for (B).

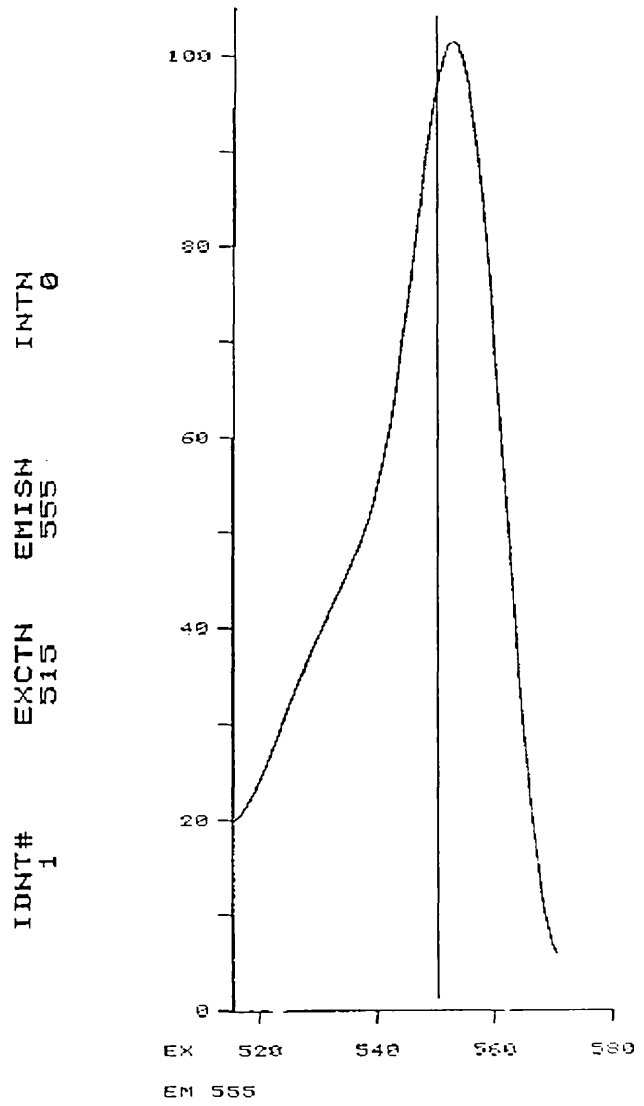
Appendix II



Representative absorption spectra from the Bioxytech LPO-586 assay kit for a sample of five decoated (mesotesta removed) *Carica papaya* L. seeds. The vertical line represents the absorbance value at which experimental measurements were made (586 nm), this was coincidental with the point of maximal absorbance.

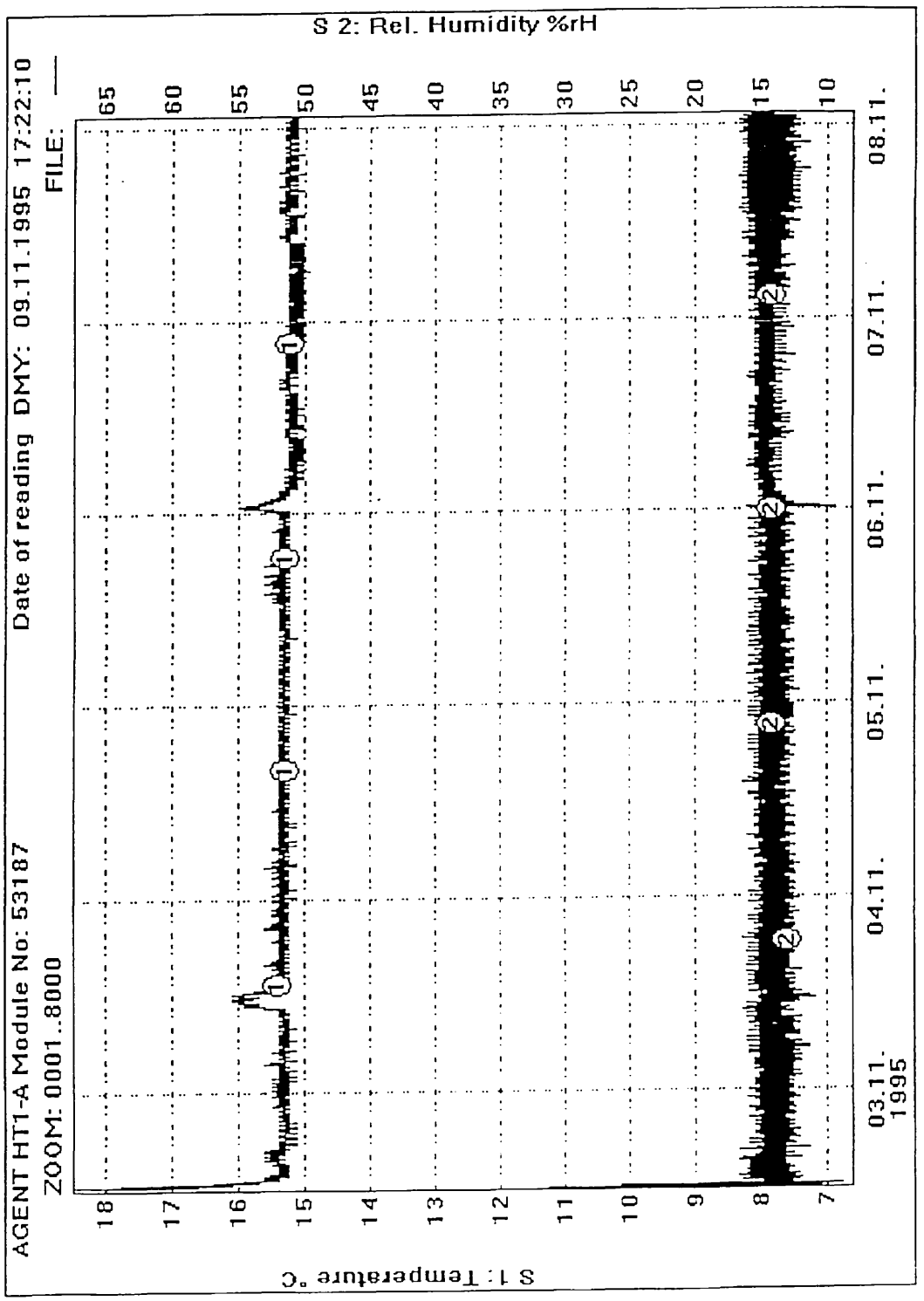


A wide scan absorption spectra obtained from the Kamiya LPO K-assay™ (LPO-CC) using the 50 nmol/ml cumene hydroperoxide standard as supplied by the manufacturer. The vertical line represents the value at which experimental measurements were made (675 nm), this was coincidental with the point of maximal absorbance.



A representative excitation spectra obtained from conducting a fluorimetric determination of TBARS on a diluted extract of horse chestnut embryonic axes. The vertical line represents the value at which experimental measurements were taken and was coincidental with the point of maximal excitation.

Appendix III



Example output from the Rologg HT1A temperature (1) and % eRH (2) monitoring unit, showing the uniformity of the 15 °C / 15 % eRH conditions of the drying room.

Appendix IV

Vs / Vc	U-525	Vs / Vc	U-525	Vs / Vc	U-525	Vs / Vc	U-525
1.00	0.00	2.00	1.00	3.00	2.18	4.00	3.57
1.05	0.05	2.05	1.06	3.05	2.24	4.05	3.65
1.10	0.09	2.10	1.11	3.10	2.31	4.10	3.73
1.15	0.14	2.15	1.17	3.15	2.37	4.15	3.80
1.20	0.19	2.20	1.22	3.20	2.44	4.20	3.88
1.25	0.24	2.25	1.28	3.25	2.50	4.25	3.96
1.30	0.29	2 30	1.34	3.30	2.57	4.30	4.04
1.35	0.33	2.35	1.39	3.35	2.64	4.35	4.12
1.40	0.38	2.40	1.45	3.40	2.71	4.40	4.21
1.45	0.43	2.45	1.51	3.45	2.77	4.45	4.29
1.50	0.48	2.50	1.57	3.50	2.84	4.50	4.37
1.55	0.53	2.55	1.63	3.55	2.91	4.55	4.46
1.60	0.58	2.60	1.68	3.60	2.98	4.60	4.54
1.65	0.63	2.65	1.74	3.65	3.06	4.65	4.63
1.70	0.69	2.70	1.81	3.70	3.13	4.70	4.71
1.75	0.74	2.75	1.87	3.75	3.20	4.75	4.80
1.80	0.79	2.80	1.93	3.80	3.27	4.80	4.89
1.85	0.84	2.85	1.99	3.85	3.35	4.85	4.98
1.90	0.90	2.90	2.05	3.90	3.42	4.90	5.07
1.95	0.95	2.95	2.11	3.95	3.50	4.95	5.16

Vs / Vc	U-525	Vs/Vc	U-525	Vs / Vc	U-525	Vs / Vc	U-525
5.00	5.25	6.00	7.32	7.00	9.93	8.00	13.31
5.05	5.35	6.05	7.44	7.05	10.08	8.05	13.51
5.10	5.44	6.10	7.56	7.10	10.23	8.10	13.71
5.15	5.54	6.15	7.67	7.15	10.38	8.15	13.91
5.20	5.63	6.20	7.79	7.20	10.53	8.20	14.11
5.25	5.73	6.25	7.92	7.25	10.69	8.25	14.32
5.30	5.83	6.30	8.04	7.30	10.85	8.30	14.53
5.35	5.93	6.35	8.16	7.35	11.01	8.35	14.75
5.40	6.03	6.40	8.29	7.40	11.17	8 40	14.97
5.45	6.13	6.45	8.42	7.45	11.34	8.45	15.19
5.50	6.23	6.50	8.55	7.50	11.50	8.50	15 41
5.55	6.34	6.55	8.68	7.55	11.67	8.55	15.64
5.60	6.44	6.60	8.81	7.60	11.84	8.60	15.88
5.65	6.55	6.65	8.94	7.65	12.02	8.65	16.11
5.70	6.65	6.70	9.08	7.70	12.20		
5.75	6.76	6.75	9.22	7.75	12.38		
5.80	6.87	6.80	9.35	7.80	12.56		
5.85	6.98	6.85	9.50	7.85	12.74		
5.90	7.09	6.90	9.64	7.90	12.93		
5.95	7.21	6.95	9.78	7.95	13.12		

The relationship between the experimental Vs / Vc ratio and the SOD activity (expressed as SOD-525 units / ml). Reprinted from the *Spectrophotometric assay of SOD activity using the SOD-525 method*, Bioxytech S.A., Bonneuil / Marne, France.

Appendix V - Statement of Advanced Studies

Advanced studies undertaken in connection with the programme of research

During the programme of research the candidate has participated in two seminars organised within the Royal Botanic Gardens and the University of Abertay, Dundee. The first of which involved a poster presentation, and the second an oral presentation was made on the role of oxidative stress and seed germplasm survival.

The candidate has also attended the following meetings which were held outside the Royal Botanic Gardens:

- Attendance at the Fifteenth Annual Seed Biology Meeting. Forestry Authority Commission, Alice Holt Lodge, Surrey, UK. 14th April, 1994

- Attendance at the 653rd Biochemical Society Meeting (Free Radicals and Oxidative Stress - Environment, Drugs and Food Additives). The University of Sussex at Brighton, East Sussex, UK. 13th-16th December, 1994

- Attendance at the Sixteenth Annual Seed Biology Meeting. Writtle College, Essex, UK. 30th March, 1995

- Attendance and poster presentation at the Fifth International Workshop on Seeds. The University of Reading, Berkshire, UK. 11th-15th September, 1995

- Attendance and poster presentation at the 657th Biochemical Society Meeting (Free Radical Processes in Plants). University of Aberdeen, Grampian, UK. 19th-21st December 1995

- Attendance and oral presentation at the Seventeenth Annual Seed Biology Meeting. University of Hertfordshire, Hertfordshire, UK. 3rd April, 1996

- Attendance of the Society of Low Temperature Biology Meeting (Analytical Techniques in Low Temperature Biology). University of Abertay Dundee, Tayside, UK. 3rd-6th September, 1996

- Attendance and oral presentation at the 2nd International Workshop on Desiccation-Sensitivity and -Tolerance in Seeds and Vegetative Plant Tissues. Franschoek Mountain Manor Hotel, Capetown, South Africa. 6th-10th January, 1997

- Attendance at post-workshop field course. Capetown Peninsula, Capetown, South Africa. 11th-15th January, 1997.

The candidates work has also been presented by colleagues at the following meetings held outside the Royal Botanic Gardens:

- Oral presentation made by Dr H. W. Pritchard at the Fifth International Workshop on Seeds. University of Reading, Berkshire, UK. 11th-15th September, 1995

- Oral presentation made by Dr R. J. Probert at the International Seed Techniques Training Course. Sao Paulo State University, Sao Paulo, Brazil. 18th June, 1996.

- Oral presentation made by Dr D.H. Bremner at Oxygen, free radicals and environmental stress in plants the 2nd International Conference held under the auspices of the Society for Free Radical Research . Don-Bosco-Haus Congress Centre, Vienna, Austria. 10th-13th September, 1996.

- Oral and written presentation made by Dr E.E. Benson at the Cryobiology Society 1996 Annual General Meeting (Spanish Interdispilanary). Experimantal apparatus to develop cryobiological methods for plant germplasm conservation. Madrid, Spain.

The candidate has contributed to monthly research group meetings and attended Kew Science Innovation lectures on a regular basis. A postgraduate presentation was made at the University of Abertay, Dundee which involved both the production of a proceedings type paper and an oral presentation. The candidate was involved with both preparations for and meetings with a Scientific Visiting Group which came to Kew between the 22nd-25th January 1996. Facilities have been accessed at the Universities of Sussex, Oxford and Wye College, University of London and relevant lectures at the Royal Society, London have also been attended.

During the course of study the candidate has enjoyed membership of the Institute of Biology, The Biochemical Society and The Society for Free Radical Research. This in conjunction with research presentations at some of the above meetings have combined to give the candidate a broad spectrum of activities during postgraduate research activity.

The published paper cited below has been removed from the e-thesis due to copyright restrictions:

(1995) Do Epr Spectra Show the Presence of a Unique and Ubiquitous Quinone-Derived Free Radical That is Associated with Senescence in Plants?, Free Radical Research, 23:2, 187-190, DOI: 10.3109/10715769509064031

Appendix VI

Refereed Papers to Arise from the Project

- **Do EPR spectra how the presence of a unique and ubiquitous quinone-derived free radical derived free radical that is associated with senescence in plants?** (1995) Goodman, B.A., Deighton, N., Glidewell, S.M., Wood, C.B., Pritchard, H.W. and Benson, E.E. *Free Radical Research Communications* **23** 187-190

- **Effects of desiccation after hydrated storage on oxidative stress in recalcitrant seeds of *Aesculus hippocastanum* L.** (1998) Wood, C.B., Pritchard, H.W., Deighton, N, Benson, E.B., Goodman, B.A. and Bremner, D.H. *Plant Physiology* (submitted).

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